THE UNIVERSITY OF LIVERPOOL

# Investigation of the MUC1-independent action of circulating galectins in metastasis promotion

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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September

2013

# ACKNOWLEDGEMENTS

I am grateful and indebted to my supervisor Dr Lugang Yu for his guidance and support throughout my research and for his constructive criticism and excellent advice during the preparation of this thesis. I am also very grateful to my co-supervisor, Professor Jonathan Rhodes for his advice throughout this period. I would like to thank colleagues from our research group, in particular, Dr Carrie Duckworth and Dr Qicheng Zhao for their technical support and advices.

I am grateful to my father, Ming Chen and my mother, Zhenhua Ma. This work would not have been possible without their financial and spiritual support.

# AUTHOR'S DECLARATION

All techniques and experiments performed and described in this thesis were undertaken by me as a student working towards the degree of Doctor of Medicine at the University of Liverpool.

Neither this thesis, nor part of it has been submitted in support of an application for another degree or qualification at this or any other university or institute of learning.

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# ABSTRACT

Galectins are galactoside-binding proteins that are expressed by various types of human cells. Recent studies have shown that the levels of circulating galectins are significantly higher in the bloodstream of cancer patients and promote cancer cell adhesion and aggregation by interaction with the cancer-associated mucin protein MUC1. However, previous studies have also shown that circulating galectins have MUC1-independent actions in metastasis promotion. This thesis further explores these MUC-1 independent actions. The studies reported here show that galectin-2, 3, -4 and -8, whose serum concentrations are all increased in cancer patients, induce secretion of various cytokines from endothelial cells *in vitro* and *in vivo*: (G-CSF, GM-CSF, IL-6 and sICAM-1 by galectin-3; G-CSF, IL-6 and GROα/CXCL1 by galectin-2; G-CSF, IL-6, GROα/CXCL1 and MCP-1/CCL2 by galectin-4 and -8). The secretion of these cytokines autocrinely/paracrinely enhances the endothelial expression of cell surface adhesion molecules, causing adhesion of cancer cells to the blood vascular endothelium. The galectin-induced secretion of cytokines is also shown to promote endothelial cell migration and tubule formation in angiogenesis. Intravenous introduction of a pathological galectin concentration into nude mice resulted in significant increase of circulating cytokine concentrations within 24 or 48 hours. Higher serum levels of these galectins correlated with higher serum levels of these cytokines in colon and breast cancer patients. Thus the increased circulation of galectins in the bloodstream of cancer patients promotes secretion from the blood

vascular endothelium of metastasis-promoting cytokines that enhance circulating cancer cell adhesion and angiogenesis. These MUC1-independent actions of circulating galectins likely make an important contribution to the metastasis-promoting action of circulating galectins. Targeting the actions of circulating galectins in cancer patients therefore represents a promising therapeutic strategy to reduce metastasis and improve survival.

# ABBREVIATIONS

| | |
|---|---|
| ACF | aberrant crypt foci |
| AGE | advanced glycation end-products |
| Akt | Protein Kinase B (PKB) |
| AP-1 | activator protein 1 |
| APC | adenomatous polyposis coli |
| BBE | Bacteroides Bile Esculin |
| BCL2 | B-cell lymphoma 2 |
| BER | base-excision repair |
| bFGF | basic fibroblast growth factor |
| BRAF | v-raf murine sarcoma viral oncogene homolog B |
| BSA | Bovine serum albumin |
| CAMs | cell adhesion molecules |
| CCL2 | Chemokine (C-C motif) ligand 2 (MCP-1) |
| CCL5 | Chemokine (C-C motif) ligand 5 (RANTES) |
| CCL20 | Chemokine (C-C motif) ligand 20 (LARC) |
| CCR | C-C motif receptor |
| CM | conditioned medium |
| CRDs | Carbohydrate recognition-binding domains |
| CXCL1 | Chemokine (C-X-C motif) ligand 1 (GROα) |
| CXCL12 | the stromal cell-derived factor 1 (SDF-1) |
| CXCR2 | interleukin 8 receptor, beta (IL8RB) |
| DCC | Deleted in Colorectal Carcinoma gene |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| ECL | enhanced chemiluminescence |
| ECM | extracellular matrix components |
| EDTA | ethylenediaminetetraacetic acid |
| EGM | endothelial growth media |
| ELISA | enzyme-linked immunosorbent assay |
| ELR | Glutamic acid-leucine-arginine |
| ERK | extracellular signal-regulated kinases |
| FAP | familial adenomatous polyposis |
| Fas | CD95 |
| FCS | fetal calf serum |
| G-CSF | Granulocyte colony-stimulating factor |
| GlcNAc | N-acetylglucosamine |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GPCRs | G protein coupled receptors |
| GROα | Chemokine (C-X-C motif) ligand 1 (CXCL1) |

| | |
|---|---|
| GTP | Guanosine-5’-triphosphate |
| H-RAS | GTPase H-Ras (transforming protein p21) |
| hEGF | human Epidermal Growth Factor |
| HMVEC-Ls | human micro vascular lung endothelial cells |
| HNPCC | hereditary nonpolyposis colorectal cancer |
| HUVEC | human umbilical vein endothelial cells |
| ICAM-1 | intercellular adhesion molecule-1 |
| IFN | interferon |
| IRF-1 | interferon regulatory factor 1 |
| IGF-1 | insulin-like growth factor 1 |
| IL-6 | interleukin 6 |
| JAK | Janus kinase |
| K-RAS | GTPase K-Ras (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) |
| KSHV | Kaposi’s sarcoma-associated herpesvirus |
| LAMP | Lysosomal-associated membrane protein |
| LARC | Chemokine (C-C motif) ligand 20 (CCL20) |
| LFA-1 | leukocyte function-associated antigen-1 |
| Mac-1 | macrophage adhesion ligand-1 |
| MAPK | mitogen-activated protein kinases |
| MCP-1 | Chemokine (C-C motif) ligand 2 (CCL2) |
| MEK | mitogen-activated protein kinase |
| MGAT5 | beta1, 6N-acetylglucosaminyltransferase V |
| MMP | matrix metalloproteinase |
| MMR | mismatch repair |
| N-CAM | neural cell adhesion molecule (CD56) |
| N-RAS | neuroblastoma RAS viral oncogene homolog |
| NER | nucleotide-excision repair |
| NF-Kappa B | nuclear factor Kappa B |
| NHEJ | non-homologous end joining |
| NIH | National Institute of Health |
| NK | natural killer cell |
| NWAG | Asp-Trp-Gly-Arg amino acid sequence |
| PAGE | Polyacrylamide gel electrophoresis |
| PBMC | peripheral blood mononuclear cells |
| PCD | programmed cell death |
| PCTA-1 | prostate carcinoma tumour antigen-1 |
| $PGE_2$ | prostaglandin E2 |
| PI | propidium iodide |
| PI-3 | phosphatidylinositide 3-kinase |
| Poly-HEMA | poly-2-hydroxyethyl methacrylate |
| PTEN | Phosphatase and tensin homolog |
| RAF-1 | Raf proto-oncogene serine/threonine-protein kinase |

| | |
|---|---|
| RANTES | Chemokine (C-C motif) ligand 5 (CCL5) |
| SDF-1 | the stromal cell-derived factor 1 (CXCL12) |
| SDS | sodium dodecyl sulphate |
| STAT | Signal Transducers and Activators of Transcription |
| TCR | T cell receptor |
| TEMED | Tetramethylethylenediamine |
| TF | Thomsen-Friedenreich oncofetal carbohydrate antigen (N-acetyl-D-galectosamine linked to protein) |
| Tn | GalNAc-α |
| TNF-α | tumor necrosis factor-alpha |
| TNM | the TNM Classification of Malignant Tumor (Tumor, Lymph Nodes, Metastasis) |
| UV | Ultraviolet light |
| VEGF | Vascular endothelial growth factor |

# TABLE OF CONTENT

# CHAPTER 1 Introduction

## 1.1 Cancer Epidemiology

In 2010, cancer caused 157,250 deaths in the UK, 28% of the total (Office for National Statistics)(1). Cancer mortality is higher in males (31%) than in females (26%). Up to 1,660,290 newly diagnosed cancer cases can be expected in 2013 in the USA, and nearly 1,600 people per day will die because of cancer(2).

Over seventy-five percent of cancer deaths occur in people more than sixty-five years old, and more than half of the cases are in people seventy-five and over. In the UK, cancers of lung, bowel, breast and prostate caused nearly half of cancer related deaths with lung cancer, which in 2010 caused around 34,900 deaths, the leading cancer cause of death (3).

The cost of cancer is enormous. In 2010, the amount spent on cancer treatment was 9.4 million pounds in the UK, and this number is expected to reach 15.3 million pounds by the year 2021, which is an increase of 62% (4). According to the American National Institute of Health (NIH), the overall cost of cancer in the USA in 2008 was 201.5 billion dollars(2).

The overall cancer mortality is decreasing despite small increases in incidence. Better survival rates have resulted from earlier diagnosis and improved treatments.

## 1.2 Cancer Biology

### 1.2.1 Genetics

At the molecular level, cancer is caused by mutations in DNA, in which process oncogenes, tumor suppressor genes and DNA repair are all involved. Cancer development generally requires mutations in multiple genes.

An oncogene is a gene that has the potential to cause cancer if mutated or over-expressed. In cancer cells, oncogenes may be inappropriately activated or abnormally expressed. The mutation of oncogenes enables the cells to undergo uncontrollable proliferation and survive, avoiding programmed cell death (apoptosis) (5). The mutation of oncogenes can result from chromosomal abnormality or single point intragenic mutations causing alterations of residues crucial to regulating the activity of the gene product. It is not necessary for both alleles of an oncogene to undergo a somatic mutation to cause an effect, such as proliferation of the cells. Since 1970, dozens of oncogenes have been reported and characterized. The mutation of *BRAF* (v-raf murine sarcoma viral oncogene homolog B) is a particularly commonly observed oncogene mutation. In cancer, valine at codon 599 may be replaced by a glutamate. The glutamate replacement simulates the structure of a phosphate group so that *BRAF* functions as a continuously activated enzyme, without requiring activation signals (6). Knowledge of oncogene changes in cancer has been used in cancer drug development to target the oncogene-encoded proteins (7-9).

Tumor suppressor genes function as a protection against cancer development. Cancer development will usually only occur once a tumor suppressor gene has mutated with resultant loss of function. The inactivation of the tumor suppressor gene could be caused by missense mutation at essential functional residues, or a deletion or an insertion that changes the gene sequence, resulting either in a truncated protein or epigenetic silencing. Tumor suppressor genes behave, unlike oncogenes, according to the “two-hit hypothesis”, which means that changes of both maternal and paternal alleles have to be mutated to have an effect, not just one (10, 11). On occasion though, a tumor suppressor gene may exhibit “haplo-insufficiency”, in which the presence of only one dysfunctional allele may be sufficient to allow cancer development (12). Some of these effects of either oncogenes or tumor suppressor genes may be mediated indirectly via effects such as altered nutrient provision or angiogenesis formation (13).

DNA repair is a process by which a cell seeks out the structural damage affecting DNA molecules and exerts a targeted fix. Structural damage to DNA is remarkably common. For each cell, up to one million molecular lesions may happen daily because of defective steps in the metabolism or external stimuli such as UV light or irradiation (14). These lesions may have harmful effects on transcriptional ability and can also cause changes in the cellular genome that persist into daughter cells after mitosis. It is crucial for a cell to keep its genomic integrity. The rate of DNA repair can be affected by internal causes such as cell type or cell age, or external causes such as nutrition supply or environmental stimulation (15). DNA repair inefficiency can directly contribute to cancer progression. The consequence of the

frequency of DNA damage and its incomplete repair is that humans would all eventually develop cancer if we lived long enough (16).

It has been shown that mutations in *BRCA1* and *BRCA2* genes, which play important roles in the DNA repair process through non-homologous end-joining (NHEJ) and homologous recombination, are strongly associated with the morbidity of breast cancer (17). In addition, epigenetic alterations in DNA repair genes, with consequent reduced production of DNA repair proteins, can also be important in the initiation of cancer (18-20). DNA repair failure in turn will also increase chances for a mutation affecting a tumor suppressor gene or oncogene (21).

In the process of chemotherapy and radiotherapy, which are the most common medical methods used in modern cancer treatment, the damage to cancer caused by therapies is aimed to be beyond the level that cells manage to repair, resulting in cell death. The therapies target cells undergoing rapid division and this provides some selectivity for cancer cells over healthy cells. However, it is inevitable that healthy cells with rapidly dividing abilities are damaged as well, such as stem cells in the bone marrow. These cells are crucial for patients' recovery after cancer therapies. Greater understanding of DNA damage and repair thus will improve our knowledge about cancer development as well as the chances of better targeted treatment and recovery (22).

Tumor stability genes, if mutated, contribute to cancer development via a different mechanism. In normal physiological conditions, stability genes, including mismatch repair gene (MMR), nucleotide-excision repair gene (NER) and base-

excision repair gene (BER), work by repairing small mistakes in the DNA molecules due to internal and external (mutagenic) causes (23).

The process of genetic changes typically occurring during cancer development is illustrated in Fig. 1.1. This process is not speedy, and may take up to 20 years to get from a normal cell to a malignant tumor. The reason it takes that long is because it requires a series of mutations to occur sequentially in the same stem cell population. In a patient who has a defective mismatch repair system it often takes a much shorter period of time, which is why patients with Hereditary Non-polyposis colon cancer develop cancer at an earlier age.

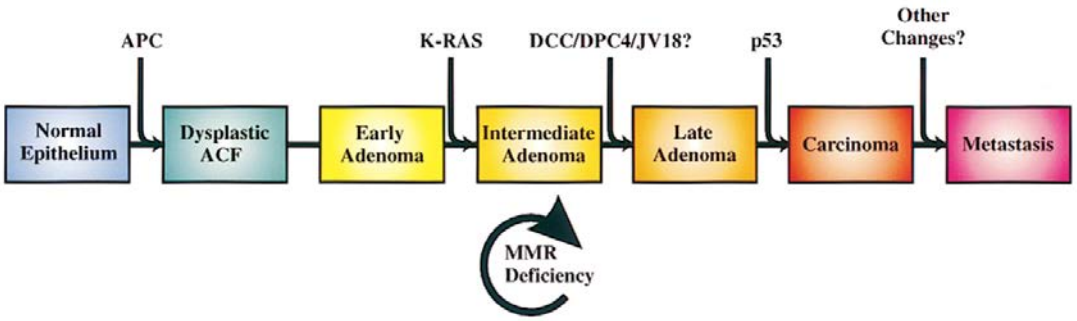


**Fig. 1.1: Genetic Changes Associated with Colorectal Tumourigenesis** (Adapted from *Kinzler et al* (24))(Permission acquired from Cell, License Number: 3345580509357)

In the above pathway, colorectal tumourigenesis starts with mutations of Adenomatous polyposis coli (*APC*) gene, followed by mutations of a series of other genes. Patients with familial adenomatous polyposis (FAP) develop numerous dysplastic aberrant crypt foci (ACF) leading to the formation of early adenomas. The oncogene *GTPase K-Ras* is activated after only one genetic event. Mutations affecting one or more of Deleted in Colorectal Carcinoma (*DCC*) gene, SMAD family member 4 (*DPC4*) gene or Mothers against decapentaplegic homolog 2 (*JV18-1*) genes on

chromosome 18q21 typically occur during progression to late adenoma with mutation of *p53* a common late event. The tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC) undergo similar (25), but not identical (26) steps of mutations. MMR deficiency speeds up this process.

### 1.2.2 Biology / Physiology

#### 1.2.2.1 Apoptosis

Cells die by one of two processes – apoptosis or necrosis. Necrosis happens when a cell is injured by an external insult that could be toxic, physical injury, infection or insufficient blood supply. Necrosis usually leads to inflammation. On the contrary, as programmed cell death, apoptosis follows a controllable, predictable routine (27). In response to apoptosis-triggering signals, caspases are activated and DNases secreted to destroy the DNA in the nucleus of the cell. The resulting apoptotic bodies are then devoured by phagocytic cells such as macrophages. Apoptosis avoids the damage that necrosis would cause (28) and plays an essential roles in metabolism. Fifty to seventy billion cells die each day via apoptosis in the adult human (29). Apoptotic cells demonstrate blebbing, cellular shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (30-32).

Increased apoptosis may lead to atrophy, while significantly reduced apoptosis

may lead to uncontrolled cell proliferation. One of the characteristics of cancer is a failure of apoptosis resulting in relative immortality of the cell line although a large percentage of cell loss from tumours is nevertheless still due to apoptosis (30). Mutations of some oncogenes interfere with apoptosis, promoting tumor formation or metastasis. Alternatively, mutations of oncogenes may promote apoptosis, forming competitive mechanisms that affect tumour development. The study of *bal-2* oncogene established the role that apoptosis contributes to neoplasia. *Bal-2* mutation inhibits apoptosis (33-35) as well as promoting lymphoproliferation and c-Myc-induced lymphomagenesis (34, 36). Fifteen B-cell lymphoma protein 2 (bcl-2) family member proteins have been identified to date, some have been proven to promote apoptosis while others have been proven to prevent apoptosis (37).

Mutations of the tumor suppressor gene *p53* are widely involved in many human cancers, and often correlate with poor prognosis (38). Elevated level of *p53* found in a myeloid leukemia cell line was strongly associated with apoptosis(39) and Injection of exogenous *p53* into nude mice provided direct evidence that *p53* could participate in apoptosis (40, 41). Many other mutations which can disrupt apoptosis are related to cancer, including Fas receptor/CD95 (42) or phosphoinositide 3-kinase (PI-3) which are activated by Ras and can be influenced by the Phosphatase and tensin homolog (PTEN) tumor suppressor (43, 44).

In spontaneously regressing tumours, the apoptosis rate has often been found to be clearly elevated. A similar situation is found when tumour cells respond to

cytotoxic anticancer agents (32). Thus apoptosis is an important target in cancer treatment.

#### 1.2.2.2 Angiogenesis

Angiogenesis means creation of new blood vessels. In the normal physiological state, angiogenesis facilitates important functions including embryonic development, repair of wounds and formation of the placenta during pregnancy.

In pathological conditions such as cancer, tumour-induced angiogenesis is indispensable for tumour development, extravasation and metastatic site formation. A tumour whose mass is larger than 0.125 $mm^2$ is too large to obtain nutrition and dispose of waste by simple diffusion mechanisms. So angiogenesis plays an essential role in remodelling the vascular tree and forming new capillaries to satisfy the new needs of the growing cancer (45). It has been suggested that primary tumours without angiogenesis may remain in equilibrium with a balance of cellular proliferation and apoptosis (46).

Angiogenesis also contributes to the process by which cancer cells enter into the blood circulation. Histological and ultra-structural analyses of tumour vessels have shown their unique properties compared to normal vessels, which include differences in cellular and basement membrane composition as well as permeability. The integrity of tumor vessels is significantly worse and easier for cancer cells to

penetrate. In addition, tumour cells induce secretion of various growth factors such as bFGF and VEGF, which accelerate the process of new capillary growth into the primary tumor site (47-49).

Angiogenesis is also necessary for metastasis. The newly formed capillaries supply oxygen and nutrients in order to support metastatic tumor expansion and angiogenesis can be considered as a rate-limiting element in the metastasis process (50-52). Angiogenesis under normal conditions as well as in cancer shares similarities in mechanism.

#### 1.2.2.3 Immunology

There is typically a “battle” between the development of a cancer and its attack by the immune system. The process of tumor immunogenicity can be divided into three stages: elimination, equilibrium and escape (53).

The first stage, elimination, happens when cancer cells draw the attention of the innate immune system often via cytokine release. Immune cells such as natural killer T cells, natural killer (NK) cells, dendritic cells and macrophages are recruited to the tumor environment.

IFN-gamma, newly synthesized by natural killer T cells and/or NK cells, directly induces tumor cell death as well as inhibiting new blood vessel formation by inducing secretion of chemokines CXCL9, CXCL10 and CXCL11, which also lead to tumor cell

death. The cell fragments produced are then ingested and eliminated by dendritic cells. The chemokines produced in this way may stimulate cytokine secretion in several parallel mechanisms and recruit more immune cells by chemotaxis. Macrophages and NK cells stimulate each other in a positive feedback loop, in which IFN-gamma and IL-12 as well as reactive oxygen and nitrogen intermediates are induced to promote tumor cell death through apoptosis. Tumor-related dendritic cells in the lymph nodes modify the behaviour of Th1 cells, which subsequently induce the development of CD8+ T cells. In turn, CD4+ and CD8 + T cells are transferred to the tumor environment, along with cytolytic T lymphocytes, thus potentially eliminating the remaining tumor cells.

If the above processes fail to clean up the tumor cells completely, the effect of lymphocytes and IFN-gamma may cause mutations which are usually unstable. In the equilibrium phase, the cells gradually obtain resistance to the human immune system. Cells that successfully acquire resistance enter the escape phase, in which they continue to grow uncontrollably (54, 55).

Various infiltrating immune cells are involved in cancer immunity and can be found in and around tumours of different kinds in different sites. (Fig. 1.2). Macrophages, mast cells, granulocytes and myeloid derived suppressor cells (MDSCs) are found in most cases infiltrating or surrounding the tumor environment both in the centre and at the diffusion edge of the tumor.

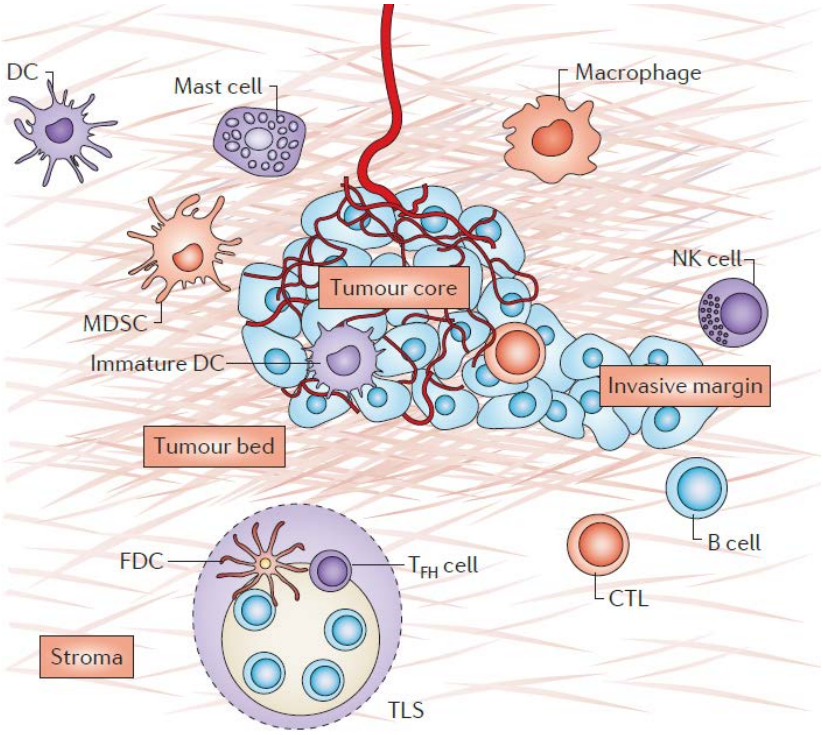


**Fig. 1.2: Cancer immunity.** (Adapted from *Fridman et al*) (56) (Permission acquired from Nature Review Cancer, License Number: 3344701138304)

Showing the cell types that may potentially interact with and attack the developing cancer

In cancer patients, immune responses (both innate and adaptive immune reactions) seem usually to be dysfunctional (57, 58). Local tumour immune escape or tumour-induced immune suppression as well as an inflammatory microenvironment are often observed. There is increasing evidence that inflammation contributes to the development of cancer and cancer also promotes the generation of an inflammatory microenvironment (59). As Trinchieri (60) stated, “the class of inflammation and immunity that is responsible for tumour initiation and early

progression is likely also to be the same class that makes the immune system unable to destroy the tumours successfully". Malignant tumours can utilise local mechanisms within their microenvironment to prevent the activation of immunological effector functions, thereby protecting the tumour from a potential immune attack (61, 62).

## 1.3 Cancer Metastasis

### 1.3.1 General

Metastasis is the main cause of cancer-associated mortality. Metastasis results from a series of sequential, interrelated steps, which involve a number of cell-cell and cell-extracellular matrix interactions (63). These include cellular transformation and growth of the original neoplasm, followed by extensive vascularisation as a consequence of angiogenesis when the primary tumour mass exceeds 2mm in diameter (64), detachment of the cancer cells and invasion into the circulation system (65), survival in the circulation, aggregation of single tumour cells to allow embolus formation, adhesion to the capillary endothelial cells (66), and eventually extravasation and proliferation at a secondary site. To continue growing, the micro-metastasis has to develop a vascular network (64), to allow adequate nutrition and oxygenation and this also allows more cancer cells to invade blood vessels (67, 68) (Fig. 1.3). A successful metastasis depends on completion of each step, so each of these steps is rate-determining (67, 68).

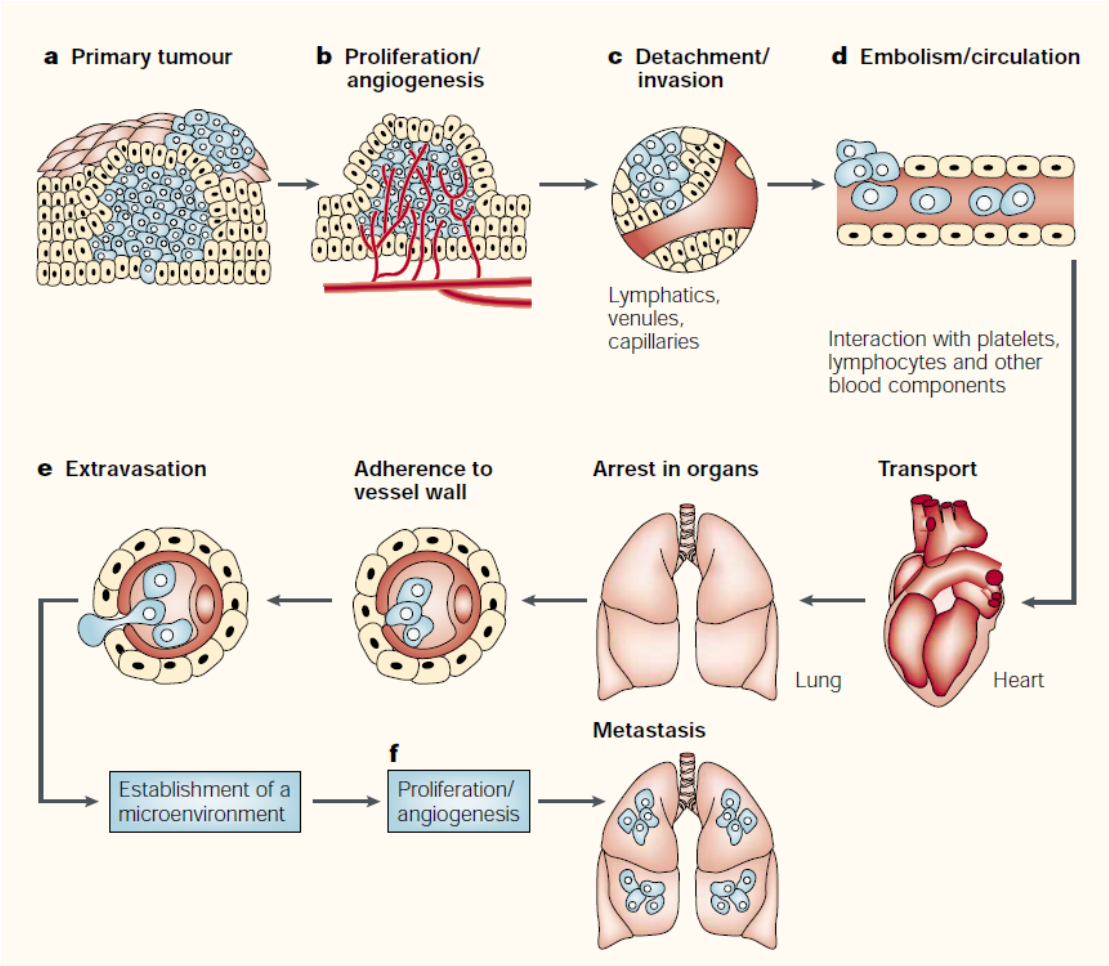


**Fig. 1.3: The main steps in the formation of a metastasis.** (Adapted from *Fidler et al* (63))

(Permission acquired from Nature Review Cancer, License Number: 3345580762453)

**a)** Cellular cancerization and development. At the initial stage of cancer formation, the nutrients required for growth can still be supplied by simple diffusion. **b)** capillary proliferation in the tumor is essential if its size is to exceed 1–2 $mm^3$ (64). The microenvironment contributes to angiogenesis by synthesis and secretion of angiogenic factors (64). **c)** continuous tumor growth results in invasion of nearby host stroma (65) through thin-walled venules, such as lymphatic channels (69, 70). **d)** Tumor cells detached from the primary tumor enter into blood circulation. Most tumor cells in the circulation are then eliminated but others may adhere to either capillary

endothelial cells or subendothelial basement membrane (66). **e)** After successful adherence at a distant site, extravasation occurs **f)** Subsequent proliferation, invasion and angiogenesis occur to support the metastasis (64) the cells from metastatic sites may enter the circulating system again to produce additional metastasis (63, 68).

The concept that formation of metastasis depends not only on the selected cancerous cells (the 'seeds'), but also relates to the microenvironments that metastatic site would be formed in (the 'soil') was first suggested in 1889 by the English surgeon Stephen Paget (71). This theory suggests that the process of tumour cells spreading to remote sites depends on the interaction of the cancer cells with homeostatic factors and with the host cells. The host cells involved include endothelial cells, epithelial cells, fibroblasts and infiltrating leukocytes.

Phenotypic and functional heterogeneity of cancer cells result from frequent genetic changes during cancer development, which in turn promote cancer metastasis by promoting proliferation, adhesion, angiogenesis and invasion (68, 72). It has been shown that 24hours after injection of tumor cells into the blood circulation of nude mice, 0.1% of the tumour cells remained alive and 0.01% of these cells survived and gave rise to metastasis (73). A "successful" tumor cell was capable of going through steps including invasion, survival in the circulation, susceptibility to immune system attack and subsequent forming metastasis site. The process of metastasis is selective for cells that succeed in all of the steps in metastasis, instead

of just a few (74). To survive all of these steps requires an apparent evolution of the cancer cells, which may cause heterogeneity within a single neoplasm. Many tumours are apparently heterogeneous, with numerous subpopulations of cells. This biological diversity, which provides different biological characteristics, including metastatic potential, could be derived of a series of gradual changes during the formation of neoplasm from a benign tumour. In other words, during neoplasia cancer cells gradually gain permanent, irreversible qualitative alterations in their characteristics. (75).

The development of experimental pathology brought scientists new knowledge of factors responsible for the formation of metastasis. By observing labelled cancer cells injected into nude mice, it was found that metastasis usually forms in not all but only some organs. It was also observed that, in the organs affected, the tumour cells were stuck in the capillaries while, in the sites where metastasis rarely occurred, circulating cells were mainly staying in arterioles (76). This provided a clue that the direction of metastasis may depend greatly on mechanical factors.

Experiments in which the relationship of blood supply and metastasis formation site were investigated have shown variable correlation. Thus a metastasis experiment using syngeneic mice indicated that although mechanical arrest does contribute to the metastasis, circulating tumor cells growing into secondary sites were also under the influence of specific organ cells (77). Comparison of carcinoma metastases in the livers and lungs of rabbits, showed that more and larger liver metastases were

observed, which was similar to that which happens in humans (78). Thus it seems that a given type of cancer cells would be attracted to specific organs. Furthermore, both cancerous cells and host cells adjust their behaviours in this microenvironment, such as altering cell-surface receptor expression or growth factor secretion (79, 80).

Thus both mechanical and organ-specific mechanisms contribute to the formation of a metastasis. Therefore, therapy for metastasis should be not only focused on the cancer cells themselves, but also against the homeostatic factors that contribute to the microenvironment.

Through the development of metastatic research, the 'seed' has now been defined as a progenitor, and the 'soil' has been defined as the host factors and microenvironment. Over more than 120 years of scrutiny and critical analysis, this 'seed and soil' hypothesis has become the prevailing viewpoint for the mechanisms behind metastasis.

### 1.3.2 Cell-cell Homotypic Aggregation

As mentioned above, in the seed and soil hypothesis, cancer cell heterotypic adhesion to blood vessel endothelium and tumour cell homotypic aggregation are two coordinated but essential parts in the metastatic process. It has been shown that in the metastasis of breast and prostate carcinoma, cancer cells form multi-cellular homotypic aggregates before they first attach to the endothelium *in vitro* and

*in vivo* [32]. Cancer cells selected *in vitro* with enhanced homotypic aggregation kinetics were shown to have a higher chance to induce metastasis compared to control cells [33-35]. Aggregated cells also showed higher viability in the blood circulation in rats [23, 24]. Injection of pre-treated DHD/K12/TRb colon cancer cell aggregates into syngeneic BS IX rats produced more than four times the amount of liver metastasis than that of single cancer cells in suspension [25].

It has also been demonstrated that the small vasculature is the place where cancer cells aggregates can arrest efficiently and more easily form metastasis [36]. Aggregation of cells increases their ability to resist anoikis (suspension-induced apoptosis) [37]. Anoikis is a special type of apoptosis that occurs to free floating cells in the blood circulation in the absence of cell-cell and cell-matrix interactions [38]. This is considered to be a vital mechanism for removing circulating tumour cells in the body [39]. Thus the formation of tumour cell aggregates represents an important step in the development of metastasis [40].

### 1.3.3 Adhesion to Endothelial cells

Metastasis is an organ-preferential process which involves the action of a variety of cell surface adhesion molecules (81, 82). For example, the regional lymph nodes, bone marrow, lung and liver are the commoner metastasis organs for breast cancer. There have been three possible mechanisms proposed in the past to explain

the organ-specific manner of cancer metastasis: circulation patterns, mechanical trapping and cell adhesion.

After cancer cells have invaded from the primary tumour into the blood or lymphatic circulation the direction and connection of lymphatic or venous drainage becomes a significant factor determining the site of metastasis however metastasis site does not always correspond to the predicted downstream site (63). Patients with different primary tumours were analysed to find out the relationship between relative blood supply and metastasis formation in a given organ. This showed that the preferred metastasis organs of breast cancer were the lungs, bones, liver and brain, with no relation to the circulatory connections to the breast. Intravenous injection of melanoma cells had also shown that, although the number of tumour cells reaching all organs are approximately even, metastasis only occurs in the lungs and ovaries, but not in the kidneys (83). Therefore, circulation patterns alone cannot explain the mechanism behind the formation of metastasis.

The mechanical trapping hypothesis suggested that the relative size of the tumour cells and tumour cell clusters play a key role in the initial site of tumour arrest (84). By acting as micro-emboli, cancer cell aggregates produce more metastases than single cell populations (85-87). However, it has also been observed that colon carcinoma cells preferentially adhere to the endothelium on the surface of lung and liver capillaries, although the diameters of the micro-vessels involved were larger than the adherent tumour cells (88). This indicates that other factors, besides

mechanical trapping, play a key role in the organ-specific metastasis of all types of cancers.

Another possible factor is the site-specifity of different cell adhesion mechanisms which involve cell adhesion molecules such as integrins, lectins and their ligands (89-91). In a recent study of breast cancer, 344 primary breast tumours of lymph node-negative patients were classified into different molecular subtypes according to the 'intrinsic' gene list describing the subtypes, in which Luminal A subtype was abundant in bone relapse but absent in lung relapse. Focal adhesion was found to be up-regulated in the luminal A subtype but down-regulated in lung relapse (92), which indicates that molecular subtypes cause preferential metastasis at different sites and that the rate of metastasis in the lung is reduced as a consequence of down-regulation of local adhesion molecules. Although the exact adhesion molecules involved in cancer cell adhesion to endothelial cells are still unclear, several families of adhesion molecules, including cell adhesion molecules (CAMs) (93) are believed to be involved.

### 1.3.4 Cytokines in Cancer Metastasis

#### 1.3.4.1 General

Cytokines are a collection of proteins, peptide or glycoproteins secreted by various cells in the human body. They serve fundamental functions involving

regulation of hormones and mediation of signalling in the inflammatory responses (94).

Most cytokines are relatively small, usually less than 20 kDa. The secretion of cytokines is typically autocrine or paracrine, but not endocrine, and is usually induced as a response to various stimuli at the transcriptional or translational level. The production of cytokines is typically transient and their constitutive production is seldom observed. Each cytokine binds to its specific cell surface receptor to perform its function, which may include gene expression alterations in the target cells. Cytokines serve a variety of functions including cell proliferation and cell differentiation and serve a particularly important physiological role in the regulation of haematopoietic cells (94-96).

The concepts of cytokines, growth factors and hormones overlap. The term cytokines was first used when associated with haematopoietic cells and the immune system. It was later realized that the some of the cytokines also contribute to cell and tissue development in other systems and to metabolism. The term “cytokine” can also be used interchangeably with the term “growth factor”. They share similarities and can act as messengers and mediators for the basic physiological functions. However, whilst some of the cytokines such as G-CSF and GM-CSF promote proliferation, others may have an inhibitory effect. Another unique characteristic is that cytokines with structurally dissimilarity can perform remarkably similar effects on different cells, and individual cytokines may perform widely different functions

(97).

Although growth factors, cytokines and hormones are very similar to each other, the production of growth factors is persistent and less tightly regulated and their major actions are usually targeted on non-haematopoietic cells. For classical hormones, the hormone is produced by specialised cells, such as insulin, which is produced by pancreas beta cells. Nevertheless, cytokines, growth factors and hormones can all function as extracellular signalling molecules. Thus receptors for certain cytokines and hormones (IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF, erythropoietin, prolactin and growth hormone) share similarities in structural features (98-104). They also share several components in their signal transduction pathways.

Chemokines form a subgroup of cytokines with the special property of chemotaxis. Responsive cells nearby can be attracted by chemokines following a signal of gradually increasing chemokine concentration to move to the sites of infection and inflammation. More than 45 chemokines are currently known. They serve fundamental functions in normal metabolism as well as in cancer.

Based on the position of the first two cysteine residues in their structure, the chemokines can be classified into four groups. The CC chemokine or β-chemokine proteins have 27 members which in structure have two adjacent cysteines near their amino terminus. Their major functions include chemotaxis of monocytes, NK cells and dendritic cells. The CXC chemokines or α-chemokines contains two N-terminal

cysteines separated by one other amino acid. Seventeen members of this family can be subcategorized into two groups based on the presence of absence of a glutamic acid-leucine-arginine (ELR) motif. ELR positive CXC chemokines, in which an ELR motif is adjacent to the first cysteine of CXC motif, induce the migration of neutrophils by interaction with chemokine receptors CXCR1 and CXCR2. The ELR negative CXC chemokines mainly have their effects on lymphocytes receptors, such as CXCL13. The C chemokines or γ chemokines only have one N-terminal cysteine and one cysteine downstream. The CXC3C chemokines or d-chemokines have three amino acids between two cysteines. Only one CX3C chemokine has been discovered - CX3CL1, or fractalkine, which is secreted and then tethered to adhesion molecules on the cell surface.

Chemokines perform their functions by interaction with specific G protein-coupled receptors containing seven transmembrane domains. Chemokines bind to and activate two or more cognate receptors, which is a unique property in the cytokine family. With their chemotaxis properties, chemokines have complex biological effects in pathological conditions, including cancer. In cancerous conditions the production and expression of chemokines is frequently abnormal. The signalling pathway through chemokine receptors is often altered as well for chemokines binding to an altered extracellular domain of the chemokine receptor may subsequently lead to a cascade of intracellular events (105). Thus chemokines contribute to primary tumor initiation, proliferation and survival, the regulation of

angiogenesis as well as cell adhesion, invasion and migration in the process of metastasis.

#### 1.3.4.2 Cytokines and Tumour Infiltration

Tumor-derived chemokines induce chemotaxis of leukocytes, such as T cells, dendritic cells, mast cells and monocytes/macrophages to the tumour microenvironment. Although immune cells may attack the tumour their presence may also promote tumour growth, angiogenesis and metastasis. For example, tumour-associated macrophages may promote tumor cell proliferation by releasing growth factors such as epidermal growth factor, or mediate angiogenesis by producing angiogenic mediators such as VEGF and bFGF (106). Tumor associated macrophages can also promote metastasis by modifying immunosuppression from the host by secretion of IL-10 and prostaglandin E2($PGE_2$), which are both potent immune regulators (107).

Expressed and secreted by most tumour cell lines, CCL2 (MCP-1) is believed to have a strong link to specific monocyte/macrophage recruitment, and has been shown to play a role in breast, ovarian, bladder, and lung cancer (105, 108, 109). The tumor associated macrophages in turn produce CCL2 in the microenvironment, forming an amplification loop for their recruitment (110). In bladder and breast cancer, CCL2 expression levels correlate with tumour stage and grade and are

therefore considered a marker of tumour aggressiveness (111, 112). Similar recruitment effects have also been observed with CCL8 (MCP-2) and CCL7 (MCP-3) (113).

Accumulating research indicates that CCL5 (RANTES) produced by tumor cells and stromal cells promotes metastasis. CCL5 plays a role in infiltration of macrophages and T cells by binding to receptors C-C motif receptor 1 and 5 (CCR1 and CCR5) on the cell surface. CCL5 acts as a prognostic marker in breast carcinoma. In the murine model, the presence of high intensity CCR5 binding protein Met-CCL5 dramatically inhibits CCL5 induced tumor formation, causing decreased size of established tumours (114).

#### 1.3.4.3 Cytokines and Neoplastic Transformation

Chemokines secreted by tumour cells attract leukocytes into their environment, which respond to their ligands and activate cytokine receptors expressed on the tumour cells, thus promoting tumor survival and proliferation by triggering a manner and site specific signalling cascade.

Cytokine-induced proliferation of tumour cells is particularly well characterised in Kaposi's sarcoma. The Kaposi's sarcoma herpes virus encodes a human G-protein coupled receptor, KSHV-GPCR, which shares high similarities with the structure of human CXCR2 (115). Overexpression of KSHV-GPCR in haematopoietic cells induces

increased proliferation and angiogenesis in mice (116). This phenomenon is due to KSHV-GRCR binding to CXCL8 and CXCL1, triggering a constitutive signal, which induces transcription via multiple transcription factors including activator protein 1 (AP-1) and nuclear factor Kappa B (NF Kappa B). In this way, virally-mediated chemokine/chemokine receptor expression promotes tumourigenesis by propagation of Kaposi's sarcoma. A point mutation of CXCR2 can also induce constitutive signalling in the absence of CXC chemokines, as an alternative process leading to neoplastic transformation (117).

#### 1.3.4.4 Cytokines and Tumour Growth

There is accumulating evidence that cytokines may directly promote growth and survival in various tumours.

The expression of IL-8 has been shown to be associated with growth of several types of cancer, including pancreatic cancer, gastric cancer, melanoma, Hodgkin's disease, breast cancer, cervical cancer and prostate cancer (118). It was found that in melanoma cells, IL-8 and GROα (CXCL1) are constitutively secreted and in turn enhance the proliferation of melanoma cells (119). Use of anti-sense oligonucleotides or neutralising IL-8 antibodies to inhibit IL-8 results in decreased melanoma cell proliferation *in vitro* (120). CXCR1 and CXCR2 are two receptors which IL-8 binds with high affinity and are both expressed on neutrophils. Neutralisation of

these two receptors in melanoma inhibits proliferation, indicating that they play an important role in tumour growth (121, 122). Also, the expression levels of CXCR1 and CXCR2 are found to correlate with invasion of melanoma (123). In other studies, neutralisation of GROα but not IL-8 inhibited the proliferation of melanoma cells, suggesting that cytokines may utilise different ligands to induce their effects in melanoma cells (124).

GROα, IL-8 and LARC (CCL20) have all been shown to promote growth of pancreatic tumour cell lines (125). By using xenograft models, it has been shown that the level of IL-8 correlates with pancreatic tumour cell severity and metastatic potential (126, 127). When PANC-1 cells are treated with IL-8, it is observed that IL-8 induces their invasion through Matrigel by inducing secretion of matrix metalloproteinase-2 (MMP2), which leads to matrix degradation (128). In ovarian cancer, tumor cells are found that are not only active for GROα, but also show high proliferation when stimulated by Stromal cell-derived factor 1 (SDF-1 or CXCL12) (129). Some prostate cancer cell lines produce IL-8 constitutively, which correlates with their growth *in vivo* (130). However, levels of both GROα and IL-8 are higher in diffuse compared with intestinal type gastric carcinoma, suggesting that cytokines induce tumor cell proliferation via complex mechanisms (131).

#### 1.3.4.5 Cytokines and Angiogenesis

CXC chemokines regulate angiogenesis and can thus promote cancer metastasis. The extent by which CXC chemokines induce angiogenesis depends upon the presence or absence of the ELR (Glu-Leu-Arg) motif, which is a potent promoter of angiogenesis at each chemokine's NH2 terminus (ELR+) (132). In corneal neovascularisation experiments, chemokines with ELR motifs were shown to be able to chemo attract endothelial cells to the tumourigenesis site and promoted angiogenesis (133, 134). On the other hand, CXC chemokines without the ELR motif were angiostatic (132). ELR+ chemokine-induced angiogenesis could be inhibited by the presence of ELR- chemokines (135-137), while similar effects were observed when ELR+ chemokines were substituted with other pro-angiogenic mediators such as bFGF (132). When the ELR motif was added to the ELR- chemokine CXCL9 by transfection, the product behaved as an angiogenic factor both *in vitro* and *in vivo*, indicating that CXC chemokines with/without ELR motif play an essential role in mediating angiogenic activity.

Angiogenesis induced by CXC chemokines is mediated through chemokine receptors on the endothelial cell surface (138, 139). Binding of CXCR2 and CXCR4 are pro-angiogenic, while binding to CXCR3 usually has angiostatic functions. All ELR-positive CXC chemokines binding receptor CXCR2 show pro-angiogenic properties, while ELR-negative chemokines CXCL9 and CXCL10 binding CXCR3 are angiostatic. These observations are important for understanding the mechanism by which chemokines contribute to angiogenesis, and the importance of the ELR

sequences(140).

#### 1.3.4.6 Cytokines and Tumour Cell Invasion and Metastasis

Cytokines interact with tumour cells stimulating the secretion of matrix degradation enzymes such as matrix metalloproteinases (MMPs) and serine/cysteine proteinases. These enzymes, in turn, help tumour cells detach from primary tumours, invade through the basement membrane and the extracellular matrix. Moreover, cytokine secretion by tumor cells may induce chemotaxis of mononuclear phagocytic cells. These may release additional cytokines and enzymes, forming an amplification loop that further promotes tumor cell invasion.

IL-8 can induce secretion of matrix metalloproteinases MMP-2 and MMP-9 from various tumor cell lines (141-144) to mediate the proteolysis of basement membranes. The presence of IL-8 in the culture medium of human pancreatic cancer cell line PANC-1 induced elevated level of MMP-2 in the supernatant as well as increased ability for PANC-1 migrate through Matrigel (141). IL-8 was also shown to be able to regulate MMP-2 activity in human melanoma cells (142). By using cDNA for IL-8 to transfect SB-2 melanoma cells, which produce small amounts of IL-8 in normal conditions, it was observed that MMP-2 levels and collagenase activity were up-regulated, and more cancer cells migrated through Matrigel. Additionally, the concentration of IL-8 was found to correlate with MMP-9 expression in human

prostate cancer cells both *in vitro* and *in vivo* (143).

Further studies have confirmed that chemokines enhance tumor cell invasion by interaction with cell surface molecules of the extracellular matrix, including integrins. Integrins are transmembrane proteins that function as adhesion molecules to mediate cell-cell and cell-matrix interactions. After treatment of ovarian cancer cells with SDF-1, the cell surface β1-integrin has been shown to be increased, leading to enhanced cancer cell adhesion to the extracellular matrix (145). In pancreatic tumor cells, SDF-1 has also been shown to induce enhanced tumor cell migration by a similar mechanism (146). SDF-1 interacted with the localized chemokine receptor CXCR4, induced an increase in the expression of β1-integrin, and subsequently induced increased adhesion of B16 murine melanoma cells to endothelial cells (147). This effect could be inhibited by the presence of anti-β1-integrin and anti-CXCR4 antibodies in the culture medium of murine B16 cells which reduced cancer cells adhesion to endothelial cells *in vitro* and prevented murine lung metastasis *in vivo*. In samples from breast cancer patients high concentrations of SDF-1 were observed in the popular sites of metastasis i.e. lymph nodes, lung, liver and bone marrow. On the other hand, low concentrations of SDF-1 were seen in organs such as brain, kidney and skin, which are rare sites for breast cancer.

Compared to breast cancer metastasis, the distribution of melanoma cells follows a similar appearance only with the difference that they metastasis to the skin as well. This difference may due to the fact that on melanoma cells, not only

receptors CXCR4 and CCR7 were expressed like that on the breast cancer cells, but also high levels of CCR10 were expressed, whose interaction with skin specific homeostatic cytokine CCL27 (ESkine) promotes metastasis (148).

#### 1.3.4.7 Cytokine Interaction Network in Cancer Metastasis

##### 1.3.4.7.1 Synergistic and Antagonistic Interaction

By using recombinant DNA techniques, the actions performed by each cytokine have been assessed. This has provided a lot of valuable information but at some distance from the real situation as most information was derived from the analysis of *in vitro* systems. Cytokines may exhibit pleiotropy and redundancy *in vivo* which is difficult to imitate *in vitro,* and cytokines could be easily diluted or inhibited by the microenvironment they are in, especially in the presence of their activators or inhibitors (149). However, in natural conditions a cell almost always encounters an environment in which various cytokines and biological active agents are included. These cytokines will perform a special action based on conditions in the current environment, so that, cells with different number and/or kind of cytokines at a time behave distinctively. One typical example of the synergistic action of cytokines is seen between TNF and IFN-γ. IFN-γ enhances cytotoxic effect of TNF on tumor cells (150, 151), potentiates production of CSF-1 and G-CSF from monocytes or lymphocytes (152), accelerates the differentiation of human myeloid cell lines (153),

promotes antiviral activity (154), and induces nitric oxide production in murine macrophages (155). TNF and IFN-γ partially target similar genes in target cells (156, 157). TNF and IFN-γ can both induce the transcription factor interferon regulatory factor 1 (IRF-1) (158-160), yet the pathways they work in are distinct (161). In HT29 colon carcinoma cell lines, a single cytokine TNFα or IFN-γ does not seem to induce alterations in cell viability even at high concentrations. Once these two cytokines existed in the same cell environment, a rapid and significant cytotoxic effect was observed, leading to cell death due to enhanced apoptosis (162, 163). Interestingly, not only can two cytokines together produce more effects than the sum of that from each cytokine (definition of a synergistic effect), but sometimes a cocktail of cytokines could induce significantly different qualities as well.

Antagonistic interactions may also occur amongst cytokines. IFNs, considered as growth-inhibitory, may have a mutually antagonistic effect when in the presence of growth stimulatory cytokines or growth factor in many types of cells (164).

Recognition of the synergistic and antagonistic interactions of cytokines helps to understand the complexities of their actions in the organism in physiological and pathological conditions.

#### 1.3.4.7.2 Stimulatory and Inhibitory Actions of Cytokines on Cytokine Production

Cytokines also have the ability to stimulate or inhibit the secretion of other cytokines. Thus, one cytokine secreted may induce secretion of several other cytokines, which perform their functions, leading to a biological response. This indirect action was demonstrated by the mitogenic action of IL-1 in murine thymocytes. IL-1 stimulates the production of IL-2 which then stimulates thymocytes proliferation (165). IL-1 and IL-2 can in turn both stimulate the production of IL-6 (166), GM-CSF (167), and chemokines (168) in various types of cells.

There is also evidence of secretion inhibitory actions between cytokines. Many immunosuppressive and anti-inflammatory actions of TGF-β are related to its ability to suppress cytokine secretion in T cells and mononuclear phagocytes (169). The extensive redundancy and pleiotropic actions of cytokines makes it often difficult to predict how a certain cytokine works in the complex biological system to produce its ultimate effect.

## 1.4 Galectins and their Roles in Cancer

### 1.4.1 General

Galectins are members of a family of lectins with specific binding to β β-galactoside-containing carbohydrates and glycoconjugates (170). Fifteen members of the galectin family have been identified in mammals.

All galectins share major sequence similarity in at least one conserved carbohydrate-binding site, or CRD (carbohydrate recognition domain) of around 130 amino acids (171). This domain folds to form a slightly bent β-sandwich with a groove on the concave side that forms the binding site. The binding site is long enough to contain a linear tetra-saccharide and it can be divided into sub-sites A to E which have their own specificity. Sub-site C has the specific affinity for the β-galactosides across the whole family, while other sub-sites provide the variable specificity for larger saccharides (172, 173).

After being synthesised on cytosolic ribosomes, galectins are translocated to the nucleus or outside the cells (174, 175) but all of the galectin members lack signal sequences required for the classical protein secretion pathway.

The galectins are believed to have a very old origin, as there are many galectin-like sequences in the genomes of creatures with long history, such as fungi, protists, and prokaryotes.

The secretion of galectins is a general phenomenon for various cell types in the

human body, including fibroblasts, ovary cells, epithelial cells, endothelial cells, dendritic cells, macrophages, bone marrow cells, T cells and B cells. Some galectins are widely distributed whilst others are mainly focused on only a few cell types (176).

Based on their structural differences, galectins have been classified into three categories: (i) Prototype galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15), which contain only one CRD in the structure. They exist as monomers or homodimers in a non-covalent form; (ii) the chimera galectin (galectin-3), containing one CRD linked to a non-lectin domain; and (iii) tandem-repeat type galectins (galectin-4, -6, -8, -9 and -12), consisting of two CRDs joined by a linker peptide which has a sequence of up to 70 amino acids (173).

Galectins show different binding preferences to saccharides with galactose in the structure (177, 178) and have higher affinities toward galactoside-terminated complex carbohydrates and glycoconjugates. The most well know galactoside-containing carbohydrates include lactose (4-(β-D-galactosido)-D-glucose) and N-acetyllactosamine (Galβ1, 4GlcNAc).

#### 1.4.1.1 Galectin Structure

It is known that galectins form dimers by self-association, or in some occasions, form higher oligomers. The formation of dimers is unrelated to the CRD and is dominated by other factors, including amino acid residues on the contact surface

when the monomer was formed.

There are three types of dimers known that galectins form: (i) through hydrophobic forces between N- and C-terminal residues of two monomers, called the "terminal" dimer. Two folded rotation axes which are nearly vertical to the β-sheets surface interact with each other. Such dimers are formed by galectin-1. (ii) The interactions of β-strands β1 and β6 from each monomer result in the "non-symmetric sandwich" dimer (179) which is found in fungal galectin-2. The electrostatic forces among residues within the convex surfaces of the monomers are responsible for its stability. (iii) The "symmetric sandwich" dimer has weaker interactions between individual's contact surfaces though the interactions are also kept by electrostatic forces.

The dimer types may be functionally different from each other. There is a theory that if free energies per residue for one type of dimer are greater to another, the existence of this dimer will dominate in solution. However, the real situation observed is quite complicated. For example, human galectin-1 consistently forms dimers in solution, even at low concentrations (2μM) when run on a native gel (180, 181). For other galectins, the situation may not be the same, for the galectin oligomeric state varies depending on conditions. Thus, at certain concentrations, galectin-5 and -7 exist as monomers in solution (182, 183), while galectin-5 also showed properties of agglutinating cells in the same time, indicative of cross-linking and subsequent self-association.

With regard to galectin-3, the N-terminal domain, rich in proline, glycine and tyrosine, greatly contributes to the formation of higher order oligomerization (184). After processing by transglutaminase (185), galectin-3 forms dimers or higher order oligomers through chemical interactions of their N-terminal domains (186), especially when the CRD of galectin-3 binds to synthetic carbohydrates on the cell surface (187). Oligomerization by the N-terminal domains results in galectin-3 forming an important pentameric state, which is unique in the galectin family (176).

For the tandem repeat galectins, galectin-4, -6, -8 and -9, the dimerization is essential for many of their functions, such as mediating cell adhesion and migration. However, even as monomers, tandem-repeat galectins already possess two CRDs, allowing them to share some similarities with prototype galectins in the dimer state, like galectin-1. Two CRDs on the galectin made them enable to bind to more than one β-galactoside containing motifs on varies of glycoconjugates, therefore mediating intermolecular cross-linking.

Self-association of galectins occurs among all galectin CRDs, and has great influence upon their functional effects. For example, it is observed that as the concentration of galectin-1 increases, galectin-1 induced mitogenicity of fibroblasts is reduced, whereas the cell division rate of HEP-2 carcinoma cells is increased (188). The reason for this phenomenon lies in the galectin-1 monomer-dimer equilibrium as determined by the concentration (180, 181). When the concentration of galectin-1 is higher than a certain threshold, the galectin-1 exists in the solution in the dimer

state, which mainly exerts a growth inhibitory activity (188). This biological effect diminishes if the galectin-1 concentration is enhanced and monomers dominate. The threshold of this alteration is about 40-80 μg/ml where the concentrations of monomers and dimers are similar.

Recognizing the structure of CRDs is important for understanding their function. The secondary structure of galectin CRDs consists of several β-strands folded in consecutive antiparallel β-sheets. There are 11 β-strands in the galectin structure. These amino acid sequences in the β-strands are generally homologous and highly conserved, especially within the motifs in the carbohydrate-binding site (189). The tertiary structure of the galectin CRD consists of two antiparallel β-sheets of five (β1-β5) and six (β6-β11) β-strands. They are arranged in a β-sheet sandwich structure with one concave and one convex surface and no helical segments. This structure of CRD is a universal characteristic among all the galectins.

#### 1.4.1.2 Galectin Functions

Due to their bivalent or multivalent binding properties, galectins tend to form arrays or lattice structures consisting of the lectins and their multivalent conjugates (190). They do not exert their functions simply by binding to specific glycoconjugate receptors. On the contrary, each of them binds to various different oligosaccharides on the cell surface or in the extracellular matrix glycoproteins (191). Based on their

glycosylation and in turn of their activation of certain glycosyltransferases, different cell types have variable sensitivities to individual galectin family members (192). The intracellular functions of galectins, however, result from their interactions with non-glycosylated nuclear and cytosolic biomolecules.

#### 1.4.1.3 Extracellular function

Galectins often bind to and cross-link glycoproteins and other glycoconjugates on the surface of various cell types to accomplish their extracellular functions (190). Many studies have shown that galectins are involved in the regulation of the apoptosis of immune cells such as T cells, which contribute to the regulation and control of immune and inflammatory responses. Thus, by binding to cell surface glycoproteins, galectin-1, -2, -3 and -9 induce T cell apoptosis. Galectins also impact on T-cell differentiation into subsets of Th1, Th2, or Th17 (193). Galectin-1 interacts with glycoconjugates on the extracellular matrix as well as those on endothelial cells (194) and T cells such as CD7, CD43 and CD45, to induce apoptosis (195). Galectin-2 interacts with cell surface molecule, leading to exposure of phosphatidylserine on the cell membrane surface in activated neutrophils, resulting in binding to lymphotoxin-α (196). This phenomenon has not been observed on activated cells (197). Galectin-7 specifically expressed on keratinocytes is involved in the process of *p53*-induced apoptosis in keratinocytes (198) and in colon carcinoma (199). High levels of galectin-10 are found in eosinophil and basophil leukocytes, whose

autogenic aggregation promotes the formation of Charcot-Leyden crystals (200).

Galectin-3 is widely expressed by epithelial cells and macrophages, and its function is mediated by pleiotropic binding to glycoconjugates including laminin, fibronectin, vitronectin, elastin, neural cell adhesion molecule (N-CAM), Lysosomal-associated membrane protein (LAMP) 1/2, and integrin $\alpha_5\beta_3$ on the extracellular matrix. Besides CD43 and CD45, galectin-3 can also bind to CD66, IgE and receptor as well as the Mac-2 binding protein on leukocytes. The level of galectin-3 is elevated in inflammatory conditions (201), and it is induced by cytokines such as CXC chemokine CXCL8 (202).

Amongst the tandem-repeat galectins, the expression of galectin-4 and galectin-6 is mainly limited to epithelial cells from the gastrointestinal tract (203). Galectin-4 binds to glycolipids in microvillus rafts, defining and stabilizing enterocytes (204). Galectin-8 is frequently found in various cancer cells (205). It has a close relationship to prostate carcinoma tumour antigen-1 (PCTA-1), which is a marker on the cell surface of prostate cancer cells (206). Galectin-8 promotes anti-metastasis by binding to cell surface ganglioside $GM_3$ (sialosyl lactosyl ceramide) (207, 208). Similarly to galectin-1 and galectin-3 (209), galectin-9 plays a role as a urate transporter to promote apoptosis in T cells (210). It also interacts with matrix type IV collagen and is chemotactic to eosinophils (211).

It can be shown by using different cell types and under various experimental conditions *in vivo*, that each galectin may perform contradictory effects, either

potentiation or suppression, contributing to inflammatory responses, compared to their behaviour *in vitro*. For instance, galectin-9 can attract eosinophils to inflammatory sites which is considered as a pro-inflammatory process. However, in the whole animal, the presence of galectin-9 reduces the inflammatory response by inducing apoptosis of immunocytes. Galectin-1 invariably induces suppression of immune response in a variety of animal models (212), while injection of endogenous galectin-3 into mouse model enhances the responses (213).

A question follows that, given that CRDs share significantly similarities in their structural folds, how can the differing functions of various galectins be explained? This could be partly due to amino acid residues within the carbohydrate-binding domain having conservation of different degrees. Thus, some of the amino acid residues are highly conserved and may not be able to interact with glycans on the surface of target cells. Competitive binding of galectins on the given glycoconjugate(s) and galectin heterooligomerization (214) may also contribute to the varied functional responses.

#### 1.4.1.4 Intracellular functions of galectins

Some of the galectins play significant roles inside the cells by interaction with cytoplasmic and nuclear proteins, thus modulating signalling pathways.

By interaction with H-Ras, intracellular galectin-1 accelerates H-Ras-GTP

phosphorylation (215). Galectin-1 enhances the binding of the C-terminal cysteine of Ras to a farnesyl moiety anchored in the intracellular membrane, thereby increasing activity of Ras. This galectin-1 effect is not mediated through its carbohydrate binding properties (216). This observation was further supported by a later study, showing that the galectin-Ras interaction is through hydrophobic prenyl-binding plane on galectins and farnesyl group on the C-terminal of Ras (217). By modelling the structure of farnesyl modified H-Ras, it has been shown that the structures of both Ras and galectin-1 have β-sheet like domain that fit together. The farnesyl group on a hydrophobic domain and a β-sheet domain on galectin-1 form a hydrophobic ternary complex (218).

Inside the cell, the binding of galectin-3 appears to be pleiotropic. The binding profile includes Ras as well as other cytosolic molecules (217). It has been demonstrated that galectin-3 interaction with Bcl-2 promotes cell growth and apoptosis (219). The interaction to Bcl-2 for galectin-3 can be inhibited by saccharide ligands, which indicates that galectin-3 participates in the interaction through its carbohydrate binding domain. Other evidence has shown that galectin-3 may also play a role in promoting tumor proliferation and progression in thyroid cancer by interaction with thyroid-specific transcription (220). Moreover, galectin-3 regulates mitochondrial integrity and cytochrome c release by interaction with synexin, which is essential for apoptosis regulation (221).

### 1.4.2 Galectins and Cancer

Each member of galectin family has its own distribution pattern. Some of the galectins are widely expressed, while others are limited to several specific organs. The expression varies under different stages of cell development in physiological conditions as well as in pathological conditions (222). The galectins show a unique manner of expression in tumor cells (204, 223-227). Galectins are often over expressed in tumor cells and a specific galectin can appear in cell types in which it is not normally expressed, including tumor cells and surrounding stroma cells. Conversely, when some cells become cancerous, the original highly expressed galectins can be down regulated. Not only the level of the galectins, but also their localisation alters during the transition from normal cells to cancer (224). These observations suggest the hypothesis that galectins play roles in several aspects of cancer development, such as tumor initiation, transformation, apoptosis as well as tumor metastasis, which includes cell adhesion, migration and angiogenesis.

#### 1.4.2.1 Galectin Effects on Tumour transformation

Galectin-1 and -3 may be critically involved in the initial stages of tumour transformation. Thus, galectin-1 is required in the process of transforming the phenotype of human glioma cells (228). Similarly, reduced levels of galectin-3 result in decreased growth rate of breast carcinoma cells and thyroid papillary carcinoma

cells in cell culture (229). On the other hand, galectin-3 cDNA transfection induces a transformed phenotype in a normal thyroid follicular cell line (230). It is shown that effects induced by both galectin-1 and galectin-3 are due to interactions with oncogenic Ras (215, 231). Oncogenic Ras proteins (H-RAS, K-RAS & N-RAS) participate in various aspects of neoplasia. They have to be anchored to the plasma membrane to perform their functions, in which galectin-1 is an important mediator to interact with the partners of HRAS. It is observed that overexpression of galectin-1 increases not only the content of Ras on the membrane, but also the process of cellular transformation (215). Galectin-1 also induces long-term activation of Raf proto-oncogene serine/threonine-protein kinase 1 (RAF1) and extracellular signal-regulated kinase (ERK) (232), indicating that the galectin-1 is involved in signalling transduction, in which transcription factors are activated to induce aberrant gene expression, leading to tumor transformation. Moreover, galectin-3 binding to Ras proteins (preferentially to K-RAS), induces the activation of signalling cascades to regulate gene expression at the transcriptional level by activation of RAF1 and phosphatidylinositol 3-kinase (PI3K) (231).

#### 1.4.2.2 Regulation of Apoptosis

Exogenously added recombinant galectins have been shown to induce cell apoptosis. Conversely, intracellular galectin-3, resulting from transfection of a cDNA encoding galectin-3, has been shown to inhibit apoptosis in several tumor cells when

exposed to apoptotic stimuli (233). The sensitivity of tumor cells to chemotherapeutic agents has been shown to be affected by galectin-3 (234, 235). It is observed that galectin-3 translocates from either cytosol or nucleus into the mitochondria when exposure to apoptotic stimuli (221) which inhibits the potential on the mitochondrial membrane, and subsequently reduces apoptosis (236). In the cell, galectin-3 exists in a phosphorylated form (237). If the Ser6 residue is replaced by alanine or glutamic acid due to mutation, galectin-3 loses its phosphorylation, along with its anti-apoptotic activity (238), indicating that phosphorylation is the essential structure modification for galectin-3 to perform its function. Phosphorylated galectin-3 is also known to regulate the mitogen activated protein kinase (MAPK) pathway (234).

Galectin-3 has an Asp-Trp-Gly-Arg (NWGR) motif in its C-terminal, which is similar to the motif on BCL2. It is the very same motif that is essential for BCL2 function as a suppressor of apoptosis (219). In galectin-3, the NWGR motif is essential for its carbohydrate-binding ability. Replacement of glycine to alanine in this motif results in reduction of anti-apoptotic activity (239). Galectin-3 also interacts with other intracellular molecules which contribute to its anti-apoptotic activity, such as synexin (annexin VII). When cells are treated with apoptotic stimuli, galectin-3 interacts with synexin to translocate to the perinuclear membranes (221). Although cytosolic galectin-3 functions as an anti-apoptosis factor, while in the nucleus galectin-3 promotes apoptosis (240).

Other galectins also show functions in regulating apoptosis. Tumour cells tend to be apoptotic when galectin-7 overexpression is observed (198, 241). It is also observed that galectin-12 at high level in a fibroblast cell line promotes apoptosis (242).

Galectins are also shown to regulate tumourigenesis by regulating cell growth. Thus, galectin-1 suppresses cell growth in an autocrine manner (243). This is further verified by the observation that in human glioma cells the inhibition of galectin-1 results in lower rate of cell growth (228). Galectin-7 transfectants induce slower growth rate in a colon carcinoma cell line (244), and both exogenous galectin-1 and galectin-7 reduce the growth of neuroblastoma cells (245, 246). Alternatively, galectin-3 acts as a positive growth regulator in some cases. It has been found that after galectin-3 transfection, the human T lymphoma Jurkat cells have a higher growth rate *in vitro,* whereas inhibition of galectin-3 results in slower growth in breast carcinoma cells and thyroid papillary carcinoma cells *in vivo* (229, 247). There are also examples showing that galectin-3 transfection induce slower growth rate in the prostate cancer cell line LNCaP *in vitro* and *in vivo* (248). A follow-up study revealed that the presence of cytosolic galectin-3 is shown to increase anchorage-independent growth *in vitro*, whilst nuclear galectin-3 reduces the growth of the cells (240), indicating that endogenous galectin-3 functions through different mechanisms depending on the subcellular localisation of the protein.

Galectins can regulate progression of the cell cycle. For example, galectin-3

inhibits the cell-cycle of breast cancer cells *in vitro* via down-regulation of cyclin E and cyclin A expression and increasing cyclin D1 expression (249). Subsequent studies have indicated that galectin-3 induces the activation of cyclin D1 by binding to β-catenin (250, 251).

#### 1.4.2.3 Effects on Cell Adhesion during Metastasis

During tumour metastasis, tumour cells invade the tissues nearby, disseminate through the blood/lymphatic circulation systems and form secondary tumours at organs distant from original sites. Various galectins contribute to this metastasis process by altering cancer cell adhesion migration, angiogenesis and escape from the immune response.

Galectins derived from the tumor cell can influence homotypic aggregation of cancer cells as well as adhesion between cancer cells and the endothelium. Galectins can either promote adhesion by directly binding, or inhibit it by steric hindrance to block the interactions between cells involved in the adhesion, subsequently disabling the cell-cell or cell-matrix interactions. Galectin-1 and galectin-3 prevent tumor cell adhesion to extracellular matrix proteins when added exogenously (252-254), while soluble galectin-8 reduces the adhesion of several cell lines to culture plates (255).

Galectins can also promote cell adhesion by forming a connection between cells or cell-extracellular matrix due to their bivalent or multivalent properties. They

promote tumour cell-cell homotypic aggregation and tumour cell adhesion to endothelial cells at distant organs in metastasis (256-258). Galectins bind integrins and several galectin-induced effects are reported to be mediated by integrins, thus s galectin-1 binds to $\alpha_7\beta_1$ integrin (259), galectin-3 binds to integrin $\alpha_1\beta_1$ (260), and galectin-8 binds to $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (255) as well as integrin $\alpha M\beta 2$ (261).

#### 1.4.2.4 Regulation of Tumour Invasiveness and Angiogenesis

The expression of galectins has been shown to influence the migration of tumor cells as well as their invasion. In breast carcinoma cells, it has been shown that exogenous galectin-3 can bind receptors on the cell surface, and results in increased migration of breast cancer cells through Matrigel (262). Conversely, in another study, exogenously added galectin-3 inhibited the migration of human colon cancer cell lines (HCT-15, LoVo, DLD-1, and CoLo201) (263). This might be due to galectin-3 recognising different cell-surface receptors. In addition, high levels of galectin-3 were found in the lung cancer cell line DLKP which exhibits increased cell motility and invasiveness *in vitro* (264). Moreover, a study using human astrocytes tumour cells has shown that the increased expression of galectin-1 is correlated with an enhanced migratory ability. When galectin-1 was exogenously added to astrocyte tumour cells, it increased the motility of the cells (265). In contrast, galectin-8 was shown to be an inhibitor of the migration of some colon cancer cell lines *in vitro* (266). It has been shown that galectin-3 increases angiogenesis *in vitro* and in mice (267). This

angiogenic activity could be a result of galectin-3 inducing the migration of endothelial cells.

### 1.4.3 Galectin-3

#### 1.4.3.1 General

Galectin-3, also known as CBP35, CBP30, Mac-2, L-29, L-31 and L-34, is one of the most studied galectin family members. It is expressed in variety of human cells, in particular epithelium and immunocytes. There are three major structural domains in galectin-3, (1) a N-terminal domain which contains a serine phosphorylation site, important for the regulation of intracellular signalling, which is unique for galectin-3 in the galectin family; (2) a repeated collagen alpha-like domain, which is sensitive to the presence of matrix metalloproteinase MMP-2 and MMP-9; (3) and a C-terminal sequence with a single carbohydrate recognition-binding domain (CRD). The Asp-Trp-Gly-Arg amino acid sequence (NWAG) on the C-terminal is responsible for the anti-apoptotic function of galectin-3 (171, 219, 268-270). Galectin-3 can form a β-sandwich shape when it complexes with lactose or N-acetyllactosamine (271). Nuclear magnetic resonance spectroscopy revealed that galectin-3 exists as a monomer in solution, and that the N-terminal section can extend by unfolding and has been found to interact with the CRD (272).

The galectin-3 N-terminal domain consists of nearly 120 amino acids. Although

this N-terminal section lacks carbohydrate-binding activity, it is still important for galectin-3 to perform its full biological activity (271). With the help of carbohydrate ligands, the N-terminal domain enables galectin-3 to form pentamers, which cross-link with glycoconjugates on the cell surface to form lattice like structures, subsequently influence the signalling pathways in cells (187). Through Tyr102 and adjacent residues, the N-terminal domain plays a role in oligosaccharide binding along with the CRD (273). It is also responsible for multimer formation, lectin binding to immobilised ligand clusters (274) and the cellular secretion of galectin-3 (275). To verify the function of N-terminal domain, experiments have shown that dissection of the first eleven amino acids of the N-terminal domain disable secretion of galectin-3 (269), while mutation of the conserved Ser6 structure results in galectin-3 losing its anti-apoptotic function (238).

The C-terminal domain of galectin-3 accommodates the whole carbohydrate-binding site (186, 270). Its structure of two folded anti-parallel β-sheets, which is observed by using X-ray crystal structure analysis, is typical compared with that of other galectins (271). The CRD, when binding to advanced glycation end-products (AGE), shows a stronger binding affinity than intact galectin-3 (276). Although galectin-3 only possesses one carbohydrate-recognition domain, it exhibits bi/multivalent binding properties (277, 278) through polymerisation. The C-terminal and N-terminal domains both participate in the formation of multimers of galectin-3 (187).

Galectin-3 is synthesised in the cytoplasm as a cytosolic protein but can be transported and expressed in multiple subcellular localisations from the cell nucleus to the cell surface, or can be secreted outside cells through a non-classical secretory pathway (175). It is shown that galectin-3 exerts different functions while in different locations. Extracellular galectin-3 mediates cell-cell and cell extracellular matrix interactions by cross-linking adjacent cells (279). Cytoplasmic galectin-3 functions as an apoptosis inhibitor by suppressing mitochondrial depolarisation and preventing the release of cytochrome C (221), while nuclear galectin-3 is shown to participate in the basic cellular functions including pre-mRNA splicing (280, 281), and in contrast to cytoplasmic galectin-3, has pro-apoptotic activity (240). The shift of galectin-3 from the nucleus to the cell cytoplasm can be observed in many types of human cancers, including colorectal (282, 283), prostate (284) and tongue (229), and closely correlates with tumour progression as stable galectin-3 gene transfection increases cancer cell invasion and promotes tumour angiogenesis *in vitro* (240).

It is shown that galectin-3 is expressed in various cells involved in the immune response and inflammation, such as macrophages, dendritic cells, eosinophils, mast cells, uterine NK cells and activated T and B cells (279). Galectin-3 induces apoptosis in T cells, such as human T leukaemia cell lines, human peripheral blood mononuclear cells (PBMC) and activated mouse T cells (285, 286). Galectin-3 activates the mitochondrial apoptotic pathway via cytochrome-c and caspase-3 by binding to CD7 and CD29 molecules (286), and galectin-3 also binds to CD45 (285).

Galectin-3 also induces monocytes to secrete superoxide anion (287) and is chemotactic to immunocytes (288). Exogenous recombinant galectin-3 increases human neutrophil (289) adhesion to laminin as well as endothelium (290). Galectin-3 was also reported to participate in the process by which macrophages devour apoptotic neutrophils, functioning as an opsonin (291). On the other hand, it has also been reported that the presence of galectin-3 inhibits T cell activation. Galectin-3 may bind to T cell receptor (TCR) glycans, forming complexes, thereby limiting the formation of receptor clustering, which is essential for TCR-mediated signalling (292).

#### 1.4.3.2 Galectin-3 Expression in Cancer

Galectin-3 has recently attracted attention in cancer both as a possible diagnostic marker and as a potential therapeutic target.

Thus, in thyroid cancer, increased serum galectin-3 concentrations are observed, particularly in patients with metastasis (293, 294). Galectin-3 has also been considered as a supplementary marker for cytological diagnosis of papillary thyroid cancer (295). Overexpression of galectin-3 has also been used to distinguish parathyroid carcinoma from benign adenoma (296). For example, in 92.3% of parathyroid carcinomas elevated galectin-3 was observed, while only 3.3% of parathyroid adenomas were galectin-3 positive. Besides, all parathyroid carcinoma with metastasis were found to have high expression of galectin-3 (297).

There is accumulating evidence that the level of galectin-3 expression correlates with tumor progression in various cancers and also correlates inversely with the degree of tumor differentiation (298). Galectin-3 expression also correlates with lymph node metastasis of colon cancer (299) and circulating galectin-3 concentrations are higher in colon cancer patients with liver metastasis (300). Galectin-3 expression correlates with tumour TNM staging in gastric carcinoma. Nuclear expression of galectin-3 in oesophageal squamous cell carcinoma correlates with the degree of invasion and inversely with differentiation while cytoplasmic galectin-3 has no such relationship (301). Increased expression of galectin-3 has been observed in the clear cell ovarian carcinomas (302, 303), renal cell neoplasms (304)as well as in mature Sertoli cells and Leydig cells of malignant testicular cancer (305), suggesting that the expression of galectin-3 was involved in differentiation.

#### 1.4.3.3 Role of Galectin-3 in Cancer Progression and Metastasis

It has been shown that galectin-3 plays a role in cancer cell viability and anoikis, adhesion, embolism, and proliferation, as well as angiogenesis in metastasis (191, 306, 307). Inhibition of galectin-3 expression has been shown to reduce the malignant potential of breast carcinoma cells (229, 247) and introduction of galectin-3 antisense transfectants decreases tumor growth in experimental models *in vivo* (229).

After detachment from the primary tumour, only a tiny population (0.1-0.01%) of tumour cells may successfully develop metastatic foci (308). Most of the circulating cancer cells die as a result of anoikis. Anoikis happens to some types of cells when they detached from and lose contact with the extracellular matrix (ECM) (279). It has been reported that increased expression of galectin-3 reduces death from anoikis when cells are exposed to apoptotic stimuli (187, 309, 310). This apoptotic regulation by galectin-3 could also promote anti-cancer drug resistance (236, 311).

Galectin-3 influences the adhesion of cancer cells to extracellular matrix (289, 290, 312) due to its binding to cell surface proteins such as laminin, Lamp I and II, IgE and Mac-2 binding protein (238, 272, 313, 314). Cell surface-associated extracellular galectin-3 induces detachment of cancer cells from primary sites by increasing tumour cell adhesion and invasion through interactions with basement matrix glycans (307). The over-expressed cell surface-associated galectin-3 in epithelial cancer cells interacts with galactoside-terminated glycans expressed on the surface of adjacent cells, leading to cancer cell homotypic aggregation and heterotypic adhesion to endothelial cells in cancer cell haematogenous dissemination (258, 315). During cancer cell extravasation, cell surface-associated galectin-3 has also been shown to stabilise the epithelial-endothelial interaction (275). Inhibition of the expression of galectin-3 in melanoma cells reduces tumour cell invasiveness and angiogenesis on collagen matrices (316), while the suppression of galectin-3

expression in metastatic human colon(317) and breast (229) cancer cells before injection resulted in significantly reduced metastasis in nude mice.

The formation of secondary tumours is significantly increased if the circulating cancer cells form emboli by aggregation with other tumour cells or host cells. Cancer cells with superior aggregation potential *in vitro* often demonstrate greater metastasis formation *in vivo* (318). By interacting with Mac-2-binding protein (319), galectin-3 stimulates the formation of multicellular aggregation [30]. Galectin-3 may also participate in tumor cell transformation through its interaction with oncogene Ras proteins (231). Galectin-3 binds to K-Ras preferentially, induces the activation of phosphatidylinositol 3-kinase (PI3K) and Raf-1, thus modifying gene expression at the transcriptional level (217, 231). Galectin-3 can also regulate the cell cycle to promote tumourigenesis (307, 320). It down-regulates cyclin E and cyclin A as well as enhancing cyclin D by interacting with β-catenin and c-Myc (251, 320). Conversely it can also up-regulate the expression of p21 (WAF1) and p27 (KIP1) to inhibit the cell cycle (249).

#### 1.4.3.4 Circulating Galectin-3

Although little is known regarding a physiological role for circulating galectin-3, recent investigations have shown that the concentration of free-circulating galectin-3 is increased up to 30-fold in the sera of patients with varies types of cancer including

breast, colorectal (321), lung (322), bladder (323), and head and neck cancers (324) and melanoma (325). Furthermore, even higher concentrations of galectin-3 are found in the serum of patients with metastasis. The source of increased serum galectin-3 has been speculated to be tumour cells as well as the peri-tumoral stromal cells (322). *In vitro,* under static as well as fluid flow conditions, cancer cells showed significantly increased adhesion to macro- and micro-vascular endothelial cells with the introduction of recombinant galectin-3 at concentrations similar to that found in the sera of cancer patients (326). This galectin-3 induced effect is at least in part due to its binding to the Thomsen-Friedenreich carbohydrate (galactose β1, 3N-acetylgalactosamine, TF) antigen, which is expressed by the transmembrane mucin protein MUC1 (327). The TF antigen is the core 1 structure of O-linked mucin type glycans whose expression is commonly increased in various types of human cancer (327).

The mucin protein MUC1 is a large and heavily glycosylated protein which is over-expressed and aberrantly glycosylated in most epithelial cancer cells (328). Unsubstituted TF antigen is often carried by MUC1, and expression of both MUC1 and TF are each independently associated with high metastatic potential and poor prognosis in several kinds of cancers, including colorectal and breast cancers (329, 330). Because of the large size of MUC1, overexpression results in the formation of a protective shield around the cell surface and prevents the adhesion of cancer cells to endothelial cells (326). Interaction with galectin-3 causes MUC1 cell surface

polarisation, leading to the protective shield breaking down and exposure of smaller cell adhesion molecules, resulting in adhesion of the cancer cells to endothelial cells. That is supported by *in vivo* experimental metastasis assays which show that pre-treatment of MUC1 positive, but not MUC1 negative, transfected human melanoma cells with recombinant galectin-3 before inoculation of the cells into immune deficient mice causes significant increase of metastasis (326).

Free circulating galectin-3 may also play a role in the inhibition of anti-tumour immunity by mediating the regulation of T cell activity (331). Free soluble galectin-3 binds to cell surface activated tumour-reactive T cells to produce immunosuppressive cytokines and induce T cell apoptosis. The concentration of galectin-3 required in these studies goes up to 25µg/ml however, which is much higher than the level seen in cancer patients. However, cell surface binding of galectin-3 often induces clustering of the receptors on the cell surface which may enhance the galectin-3 binding affinity up to 10,000-fold (285, 327, 332, 333). Therefore, the high concentration required for driving tumour-reactive T cell activation and apoptosis could be achieved, and could be a factor in the avoidance of tumour cell destruction by immune surveillance.

## 1.5 Glycosylation Changes in Cancer

### 1.5.1 General

Glycosylation is a fundamental cellular process that occurs in all cells. By the actions of enzymes, saccharides are joined with proteins, lipids or other biological molecules to form glycoconjugates. In cells, carbohydrates serve as signalling molecules or as structural components, which are especially important for mediating and/or modulating interactions between cells and one another or with the matrix. Glycosylation is initiated within the rough endoplasmic reticulum (ER) but continues in the Golgi apparatus.

### 1.5.2 O- and N-linked Glycosylation

It is estimated that more than half of mammalian proteins are modified by glycosylation with *O*- or *N*-linked glycans (334). *N*-linked glycosylation occurs on asparagine in the sequence Asn-X-Ser/Thr, and follows pre-synthesis of a dolichol-oligosaccharide precursor as well as other process in the ER. *N*-linked glycans typically contain glucose, mannose, and *N*-acetyl glucosamine (GlcNAc) molecules, with the initial linkage to asparagine occurring via *N*-acetylglucosamine. N-linked glycosylation usually occurs on membrane-associated and secreted proteins in eukaryote cells (335). N-linked glycans share a common branched tri-mannosyl core Man3GlcNAc2-Asn, which is essential for the formation of a diverse number of

heterogeneous, often quite large and bulky, bi-, and tri- or tetra-antennary glycans (334).

O-linked glycosylation occurs in many cell membrane and secreted proteins in the later stages of protein processing. The O-linked glycans tend to be smaller and less branched as well as more diverse than N-linked glycans. As one of the most common glycans, O-glycans are defined by GalNAc monosaccharide α-linked to the hydroxyl group of serine or threonine residues. Mucins, secreted onto the apical surface of epithelial cells, are heavily *O*-glycosylated and owe much of their protective effects to the *O*-glycans. The *O*-linked glycans on membrane-bound or secreted mucins typically comprise up to 50% of the mucin molecular weight (336) in epithelial cells, leukocytes and vascular endothelial cells (335, 337). Glycans modulate various aspects of the proteins that they attached to. For example, *O*-glycans can confer protease resistance of the proteins by protecting thermolysin-sensitive regions of the polypeptide (338).

At least eight different core structures have been reported to occur on mucin type (*O*-linked) glycans. All of these core structures are based on the core-GalNAc residue, which is further substituted at C3, C6 or at both positions with β-galactose (Gal) at C3, β-GlcNAc at C3 and/or C6, and α-GalNAc at C3 or C6 (339). This results in the formation of either core 1, core 2 or core 3 structures. Some of the mucin-type *O*-linked glycans are cancer-associated antigens, this includes GalNAc- (Tn), sialyl-GalNAc (sialyl-Tn) and Galβ1, 3GalNAc- (Thomsen-Friedenreich or TF) antigens (340).

Some of these carbohydrate antigens have been studied as therapeutic targets for cancer treatment (341).

### 1.5.3 Molecular Mechanisms of Altered Glycosylation in Cancer

In cancer cells, changes in cellular glycosylation are common. Alterations of *N*-glycosylation in tumours include the increased expression of β1-6 branched complex-type sugar chains (342) and the increased expression of the three β-*N*-acetylglucosaminyltransferases; GnT-III, -IV and –V have all been reported in cancer cells (343).

The molecular mechanism of the changes in *O*-linked glycosylation in cancer are probably much more complex. Cancer cells normally carry shorter and less branched *O*-linked carbohydrate chains, with increased sialylation and reduced sulphation (344-346). At first, altered expressions or activities of the relevant glycosyltransferases were thought to be the primary reason for altered *O*-linked glycosylation in cancer. Brockhausen et al. found that many glycosyltransferases activities were altered in epithelial cancers (347). Thus the activity of a sialyltransferase (CMP-sialic acid Gal β 1-3GalNAc α 3-sialyltransferase) was increased 4-fold in three breast cancer cell lines: BT20, MCF-7 and T47D. The Gal-β 1-3GalNAc (GlcNAc to GalNAc) β 6-GlcNAc-transferase activity was found to be deficient in mammary cancer cell lines BT20 and T47D but expressed in MCF-7 breast cancer cells

(347).

Tn (GalNAcα-Ser/Thr), sialosyl-Tn and TF (Galβ1-3GalNAcα1-Ser/Thr) antigens are often overexpressed in cancer cells. The Galβ1-3GalNAcα1-Ser/Thr structure is revealed in many human cancer cells (348, 349) when the originally attached sialic acids, sulphates or other chain structures were removed (350). However, the levels of the relevant glycotransferases that control the biosynthesis of these glycans are similar in paired specimens of normal and cancerous tissues (351). Furthermore, the enzyme activities of the glycosyltransferases do not change when the location, stage or histological type of the tumour changes in cancerous tissues (351). This indicates that the expression of glycosyltransferases alone may not account for the altered glycosylation changes in cancer cells.

It is shown that redistribution of the glycosyltransferases in the Golgi apparatus may have some relevance to the abnormal glycosylation of proteins (352, 353). In cancerous conditions, the level of core 2 β1,6-GlcNAc-transferase is greatly decreased, leading to reduced expression of core 2 structure as well as increased expression of core 1 structure, which include the TF antigen (347, 354). The increased TF expression could also be induced by enhanced activity of nucleotide sugar substrate UDP-galactose for core 1 β1, 3 Gal-transferase (355). Another mechanism that result in increased TF expression can due to alteration in intra-Golgi pH (356-358). Elevation of the Golgi pH by treatment of the cells with bafilomycin A or pH calibration buffers increases TF antigen expression in breast and colorectal

cancer cells (356, 358) and similar Golgi disarray has been shown to occur in human cancer cells (359).

### 1.5.4 Roles of Altered Glycosylation in cancer

Alteration of cellular glycosylation is a common characteristic of human cancer (360-362). Amongst the commonest glycosylation changes are the increased occurrence of GalNAcα1-Ser/Thr (Tn antigen), Neu5Acα2-6GalNAc (sialyl-Tn antigen) and Galβ1-3GalNAcα1-Ser/Thr (TF or T antigen)(363). Overexpression of sialyl-Tn has been shown to be related to resistance of the cancer cells to chemotherapy (364). In experimental mouse models, the inhibition of sialylation (365) and enhancement of mucin glycosylation (366) reduces cancer cell metastasis. Beta 1-6 branched *N*-linked oligosaccharides are consistently increased in neoplasia of human breast and colon and this has been shown to correlate with the pathological staging (367). Golgi β1, 6N-acetylglucosaminyltransferase V (MGAT5) is required in the biosynthesis of β1, 6GlcNAc-branched N-linked glycans, and tumour growth and metastasis were inhibited in Mgat5-deficient mice (368). At the cell surface, the increased expression of highly branched *N*-glycans is correlated with tumour formation and alters adhesive properties of tumour cells *in vivo* (369). It has been suggested that the increased expression of cell surface *N*-glycans contributes directly to reducing cell-cell contact as well as cell growth in an immortalised lung epithelial cell line (370).

Sialyl-Lewis$^{a}$ and Sialyl-Lewis$^{x}$ [NeuAcα2-3Galβ1-4/3(Fucα1-3/4) GlcNAc-R] are two tetra-saccharide carbohydrates that contribute to the cell-cell interaction when present on cell surface *O*-glycans. Overexpression of sialyl-Lewis$^{x/a}$ have been used as tumour markers in human adenocarcinoma of the colon (371, 372) and breast cancer (373). It has been shown that increased expression of Sialyl-Lewis$^{x/a}$ on the cell surface correlates with the metastatic potential of both human colon and stomach cancer cells (343, 374-377). The precursor of Sialyl-Lewis$^{x}$ is α2, 3-sialylated galactose residue of GlcNAc, whose N-acetylgalactosaminylated form is the precursor of Sd$^{a}$ antigen. The Sd$^{a}$ carbohydrate [GalNAcβ1-4(NeuAcα2-3) Galβ1-4GlcNAc] is a blood group structure expressed on glycolipids and glycoproteins, which can be found in the normal gastrointestinal mucosa. It is reported that the level of Sd$^{a}$ as well as the enzyme β1, 4-N-acetylgalactosesaminyltransferase (β1, 4GalNAcT) which is responsible for its synthesis is remarkably reduced in cancer lesions (378, 379). Through the competitive activity of glycosyltransferase Sd$^{a}$-β1, 4GalNAcT, Sd$^{a}$ determinant efficiently eliminated the presence of sialyl Lewis$^{x/a}$, therefore abolished their role of promoting metastasis (380).

### 1.5.5 Interactions between TF antigen and Galectin-3

The Thomsen-Friedenreich (Galβ1, 3GalNAcα-, TF) antigen is a disaccharide comprised of galactose linked to *N*-acetylgalactosamine (GalNAc), present as a core structure *O*-linked to serine/threonine. It behaves as an oncofetal antigen,

overexpressed in normal foetal tissue and in cancerous or precancerous tissue (350). It is absent or masked by further glycosylation in healthy tissues, while expressed in many cancers.

It has already been discussed that elevated levels of galectin-3 in the blood circulation promote cancer metastasis in an animal metastasis model (326). This action of galectin-3 is partially attributed to its interaction with the oncofetal TF disaccharide on the transmembrane mucin-type protein MUC1 expressed on cancer cells (327). The galectin-3-MUC1 interaction induces MUC1 polarization on the cancer cell surface leading to exposure of the underlying adhesion molecules, thereby resulting in increased tumour cell heterotypic adhesion to blood vascular endothelium and tumour cell homotypic aggregation in the circulation (381). The presence of TF antigen on MUC1 expressed on the cell surface paves the way for galectin-3 to exert its function of enhancing the adhesion between cancer cells and endothelial cells. This is a mechanism by which TF expression, a most common glycosylation alteration in cancer, may promote cancer metastasis (Fig. 1.4).

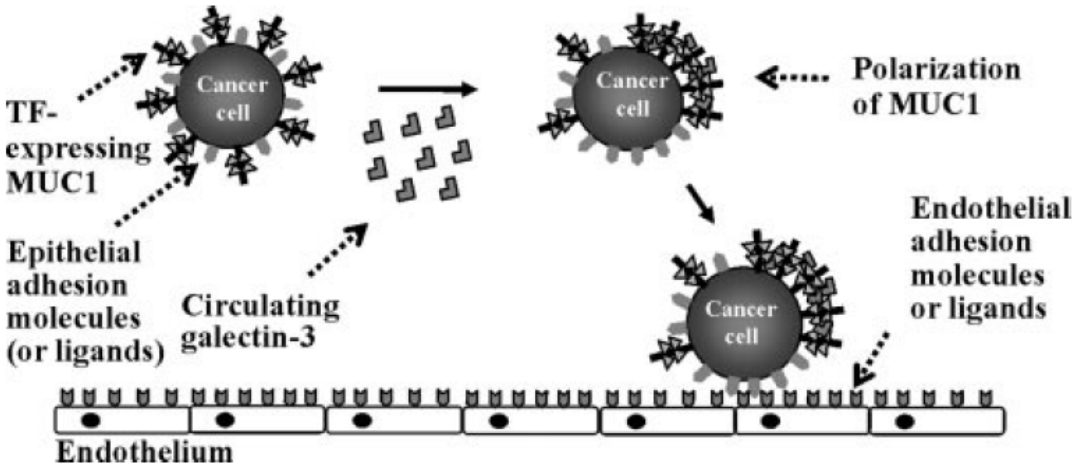


**Fig. 1.4: Proposed action of galectin-3-MUC1 interaction.** (Adapted from *Yu et al*)(327) (Permission acquired from American Society for Biochemistry and Molecular Biology)

Circulating galectin-3 interacts with cancer associated, TF antigen-bearing MUC1 on the cancer cell surface, leading to the polarization of MUC1 molecules. The subsequent exposure of smaller cell adhesion molecules or ligands set the stage for further epithelial-endothelial interaction via ligands such as E-Selectin and CD44H.

# CHAPTER 2 Hypothesis and Aims

## 2.1 Hypothesis

Circulating galectin-3, whose concentration is commonly elevated in the bloodstream of cancer patients, has a MUC1-independent, as yet unidentified action that promotes metastasis.

## 2.2 The aims of this study

The aims of this study are to identify and characterize the MUC1-independent actions of circulating galectin-3 in metastasis promotion. This will increase our understanding of the molecular regulation of metastasis and help in the identification of new therapeutic strategies to reduce metastasis.

# CHAPTER 3 Materials and Methods

## 3.1 Materials

All cell culture flasks, multi-well plates, chemicals of analytical grade and non-enzymatic cell dissociation solution (C1544-10ML) were purchased from Sigma-Aldrich Ltd (Poole, UK), unless otherwise stated.

Calcein AM (1mg/ml solution in anhydrous DMSO, C3099) was purchased from Invitrogen, Paisley, UK.

Human Cytokine Array Panel Array Kit (ARY005), Human Phospho-Kinase Array Kit (ARY003), human cytokine ELISA kits (G-CSF: DY214, GM-CSF: DY215, IL-6: DY206 and ICAM-1: DY720), Quantikine Mouse sICAM-1 (MIC100), recombinant galectins and anti-galectin antibodies were purchased from R&D Systems, Abingdon, UK.

Human cytokine ELISA kits (G-CSF: 900-K77, GM-CSF: 900-M30, IL-6: 900-M16, ICAM-1: 900-M464, GRO/MGSA: 900-K38, MCP-1: 900-M31) and murine cytokine ELISA kits (G-CSF: 900-K103, GM-CSF: 900-K55, IL-6: 900-M50) were purchased from Peprotech, London, UK.

Mouse KC (GRO-α/CXCL1) (PK-EL-65420DM) ELISA Development Kit was purchased from PromoKine, Heidelberg, Germany.

RayBio antibody MMP array-1 (AAH-MMP-1-4) was purchased from Insight Biotechnology, Wembley, UK.

Anti-integrin $\alpha_5\beta_1$ (ab25076) was purchased from Abcam, Cambridge, UK.

*In Vitro* Angiogenesis Assay Endothelial Cell Invasion Kit (3471-096-K) and *In Vitro* Angiogenesis Assay Tube Formation Kit (3470-096-K) were purchased from AMS Biotechnology (Europe) Ltd, Abingdon, UK.

## 3.2 Cell Lines

ACA19$^-$: MUC1 transfection of human melanoma A375 cells with full-length cDNA encoding MUC1 and the subsequent selection of the MUC1 negative transfectants cultured in Dulbecco's modified Eagle's medium. It was kindly provided by Dr. John Hilkens (Netherlands Cancer Institute) (382).

HCT116: Human colon carcinoma cells were from the European Cell Culture Collections via the Public Health Laboratory Services (Porton Down, Wiltshire, UK)(383). HCT116 cells were MUC1-negative malignant cells isolated from a male with colonic carcinoma and cultured in McCoy's 5a medium.

HMVEC-Ls: Human micro vascular lung endothelial cells (HMVEC-Ls) were obtained from Lonza (Wokingham, UK). HMVEC-Ls cells were cultured in EBM-2 endothelial growth media. Fewer than five passages of the cells were used in all the experiments. The culture medium was changed every four days.

HUVEC: Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Wokingham, UK) and cultured in EGM endothelial growth media (Lonza).

## 3.3 Cell Culture Medium

(1) Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich

Complete culture medium contains 10% FCS (fetal calf serum), penicillin 100 U/ml, streptomycin 100 μg/ml, glutamine 2mM.

Serum-free DMEM contains 0.5% bovine serum albumin, penicillin 100 U/ml, streptomycin 100 μg/ml, glutamine 2mM.

(2) McCoy's 5a from Sigma-Aldrich

Complete medium contains 10% FCS, penicillin 100U/ml, streptomycin 100 μg/ml, glutamine 2mM.

Serum-free McCoy's contains 0.5% bovine serum albumin, penicillin 100 U/ml, streptomycin 100 μg/ml, glutamine 2mM.

(3) EBM-2 from Lonza

Complete EBM-2 contains fetal bovine serum (BSA) 25ml/500ml, hydrocortisone 0.2ml/500ml, hFGF-B 2ml/500ml, VEGF 0.5ml/500ml, $R^3$-Insulin-like growth factor-1 0.5ml/500ml, ascorbic acid 0.5ml/500ml, hEGF 0.5ml/500ml and GA-1000 (Gentamicin Sulfate and Amphotericin-B) 0.5/500ml.

Serum-free EBM-2 contains 0.5% bovine serum albumin, hydrocortisone 0.2ml/500ml, hFGF-B 2ml/500ml, VEGF 0.5ml/500ml, $R^3$-Insulin-like growth factor-1 0.5ml/500ml, ascorbic acid 0.5ml/500ml, hEGF 0.5ml/500ml and GA-1000 (Gentamicin Sulfate and Amphotericin-B) 0.5/500ml.

(4) EBM from Lonza

Complete EBM contains Fetal Bovine Serum 10ml/500ml, hydrocortisone 0.5ml/500ml, hEGF 0.5ml/500ml, GA-1000 0.5/500ml, BBE 2ml/500ml.

## 3.4 Cell Counting

Cell numbers were counted with a haemacytometer. The number of cells was obtained with the following equation: cell density (cells/ml) = number of cells per grid square X $10^4$.

## 3.5 Cell Culture

All cells were grown as monolayers. ACA19$^-$ cells were grown in Dulbecco's modified Eagle's medium (DMEM); HCT116 were cultured in McCoy's 5a medium; HMVEC-Ls were cultured in EBM-2 endothelial growth media and supplements and HUVEC were cultured in EBM and supplements. Cells were maintained at 37℃ in a humidified atmosphere of 5% $CO_2$, 95% air. ACA19$^-$ and HCT116 cells were released from T25 $cm^2$ flasks with 1ml Trypsin (0.5mg/ml in sterile PBS) and routinely passaged at a 1:6 subculture ratio when they had become 60%-80% confluent. Cells at a ~80% confluence were used for all experiments. HMVEC-Ls and HUVEC cells were released from T25 $cm^2$ flasks with 1ml Trypsin (0.5mg/ml in sterile PBS) and routinely passaged at a 1:3 subculture ratio when they had become 60%-80% confluent. Cells at ~80% confluence were used for all experiments.

## 3.6 Cell Thawing and Plating

The cell culture medium was warmed up to 37℃ in the water bath (about 20 minutes) before use. The freezing vial of cells from the liquid $N_2$ cell bank was warmed up to 37℃ in the water bath. The cell suspension was then added to 10ml of pre-warmed cell culture medium in a 15ml tube. (To defrost HMVEC-Ls and HUVEC cells, the cells were added directly into one or two T25 cell culture flasks.) After centrifugation of the cell suspension for 5 minutes at 1000 (**g*), the supernatant was discarded. The cell pellet was resuspended in 10ml pre-warmed cell culture medium and seeded into a T25 cell culture flask. The cells were incubated at 37℃ in a humidified atmosphere of 5% $CO_2$, 95% $O_2$. The culture medium was changed every four days.

## 3.7 Detachment of the Cells with Trypsin or Non-enzymatic Cell Dissociation Solution

Sub-confluent cells in T-25 cell culture flasks were rinsed with 10ml each time PBS before 1ml Trypsin (with 0.5% porcine Trypsin and 0.2% EDTA) or 2ml non-enzymatic cell dissociation solution (NECDS) was added into each T25 flask for 5 to 10 minutes at 37℃ until the cells became detached from the flasks. After addition of 10ml pre-warmed culture medium, the cell clumps were dispersed by repeated aspiration of the cell suspension 10 times using a 10ml pipette, to produce a dispersed cell suspension.

## 3.8 Cell Adhesion Assay

HMVECs were released from the culture flasks by trypsinization and suspended at 2 x $10^5$/ml before application of 200µl/well to each well to be used in 96-well plates. The medium in each well was replaced with 200µl/well fresh EGM-2 medium after three hours in order to remove residual Trypsin. The cells were incubated at 37℃ for one day for the formation of the cell monolayers. The HMVEC-Ls monolayers were treated with or without 1µg/ml of galectin-3 or BSA in the presence or absence of lactose (100µM) for 1 hr or 24 hrs. The HMVEC monolayer was then washed with PBS and used for subsequent assessment of cancer cell adhesion.

The HMVEC cells used for obtaining the conditioned medium were released from the culture flasks by trypsinization and suspended at 2 x $10^5$/ml with EBM-2 culture medium before application of 2ml/well to each well to be used in six-well plates. The medium in each well was replaced with fresh EGM-2 medium after three hours in order to prevent the harmful effect Trypsin could have. The cells were incubated at 37℃ for one day for the formation of cell monolayers before they were treated with 1µg/ml of galectin-3 or BSA in the presence or absence of lactose (100µM) for 1 hr or 24 hr. The conditioned medium from each well was collected for future use.

The ACA19$^-$ and HCT116 cancer cells used for acquiring the conditioned medium were detached from the culture plates by trypsinization before their confluence reached 80%. They were suspended at 1 x $10^6$/ml with serum-free DMEM medium

before application of 2ml/well in six-well plates in which each well to be used was coated with Poly-HEMA 3ml/well twice. The cells were incubated at 37℃ for one day before 1μg/ml galectin-3 or BSA was added to each well. After 1 hr or 24 hr treatment, the conditioned medium from each well was collected.

The ACA19$^-$ and HCT116 cancer cells used for assessment of cell adhesion were detached from the culture plates with NECDS before their confluence reached 80%, which released the cells from the culture flasks while still keeping the cell surface proteins intact, washed and resuspended at 5x10$^6$cells/ml in serum-free DMEM medium. The ACA19$^-$ and HCT116 cancer cells were labeled with 10μl/ml Calcein AM Cell Labeling Solution at 37℃ for 30 minutes.

The ACA19$^-$ and HCT116 cancer cells were washed and resuspended with obtained conditioned medium or serum-free DMEM medium at concentration of 5x10$^4$/ml before application (5x10$^3$/well) to the HMVEC monolayer for 1 hr at 37℃. The HMVEC monolayer was washed twice with PBS and the endothelial cell-associated fluorescence was measured with a TECAN infinite F200 fluorescent microplate reader with Excitation Wavelength 485nm, Emission Wavelength 535 nm, Excitation Bandwidth 20nm, Emission Bandwidth 25nm and Gain 50 Manual.

## 3.9 Electrophoresis and Lectin/Immunoblotting

Up to 80% confluent cells in a T25 flask were washed with PBS 10ml twice. A 1ml/T25 flask SDS (sodium dodecyl sulfate) – sample buffer (see below) – was added

and incubated for 20 minutes at room temperature. The cell lysates were collected in a 1.5ml tube and kept at -20℃ until use.

**Table 3.1 SDS- sample buffer:**

| | **2 fold** | **4 fold** |
|---|---|---|
| **Stacking buffer** | 2.5ml | 0 |
| **Glycerol** | 1.0ml (20%) | 2ml |
| **Mercaptoethanol** | 0.5ml (10%) | 1ml |
| **20% SDS** | 1.0ml (4%) | 2ml |
| **1% Bromophenol blue** | 50µl | 0.1ml |

**Table 3.2 Separating gel preparation:**

| **Separating gel** | **4%** | **7.5%** | **10%** | **15%** |
|---|---|---|---|---|
| **Deionized water** | 6ml | 4.85ml | 4.2ml | 2.35ml |
| **1.5M Tris-HCl resolving gel Buffer** | 2.5ml | 2.5ml | 2.5ml | 2.5ml |
| **10% (w/v) SDS** | 100µl | 100µl | 100µl | 100µl |
| **30% acrylamide/bisacrylamide** | 1.35ml | 2.5ml | 3.33ml | 5ml |
| **TEMED** | 5µl | 5µl | 5µl | 5µl |
| **10% ammonium persulfate** | 65µl | 50µl | 50µl | 50µl |

**Table 3.3 Stacking gel preparation:**

| **Stacking gel** | **3.75%** | **4.00%** |
|---|---|---|
| **Deionized water** | 3.09ml | 3.05ml |
| **0.5M Tris-HCl stacking gel buffer** | 1.25ml | 1.25ml |
| **10%(w/v)SDS** | 50µl | 50µl |
| **30% acrylamide/bisacrylamide** | 0.625ml | 0.665ml |
| **TEMED** | 10µl | 10µl |
| **10% ammonium persulfate** | 65µl | 50µl |

After the glass plates and spacers (1.0mm thick) were assembled, the running gel was poured to about 1cm below the wells of the comb (~4.9ml). Then 80µl water-saturated butanol-1 was added on top of the gel (added from the corner of the gel). When the gel had set (~ 40 minutes), the butanol was poured off and the gel was rinsed with about 2ml deionized water three times. Stacking gel was added and the 1.0mm thick comb inserted immediately. When the stacking gel had set, the glass plates were placed in a gel rig and immersed in buffer. Prior to running the gel, the wells were flushed out thoroughly with running buffer.

**Table 3.4 Running buffer preparation:**

| **Running buffer:** | |
|---|---|
| **Tris-Base** | 30.67g |
| **Glycine** | 64.04g |
| **SDS** | 2.2g |
| **Make up with dd$H_2O$** | 4L |

Running the gels

The samples were heated at 100℃ for 10minutes before being loaded onto the gels.

Then the protein was transferred to nitrocellulose membrane

**Table 3.5 Transfer buffer preparation:**

| **Transfer buffer** | |
|---|---|
| **Tris-Base** | 12.12g |
| **Glycine** | 57.65g |
| **Methonal** | 800ml |
| **Make up with dd$H_2O$** | 4L |

The gel was sandwiched as negative pole (black) - sponge – filter paper – gel – nitrocellulose membrane – filter paper – sponge – positive pole (white) and transferred at 100V for 1 hr.

After electrode transfer, the nitrocellulose membrane was stained with Ponceau S (0.1% Ponceau S in 1% acetic acid) solution for 2-3 minutes to visualize the protein bands. To get a clearer view of the bands, the blots were washed several times with 1% acetic acid. The Ponceau staining was removed by washing the blot several times with PBS.

Immunoblotting

The membrane was blocked in blocking buffer (1% BSA in 1% TWEEN20 in PBS) for 30 minutes at room temperature or 4℃ overnight.

The first antibody in 1% BSA in TWEEN20 in PBS was then applied to the membrane according to the concentrations specified in the results section for 1 hr at room temperature. The blot was washed three times with 1% TWEEN20 in PBS 100ml/time with rolling on a rolling machine for 10 minutes each time. After removal of the solution, the secondary antibody diluted in 1% BSA in TWEEN20 in PBS was applied for 1 hr at room temperature. After the membrane was washed 3-5 times with 1% TWEEN20 in PBS 100ml each time, the binding was visualized by means of enhanced chemiluminescence (ECL) through Bio-RAD Image Lab™ and Bio-RAD software version 2.0.

## 3.10 Human Cytokine Array

All reagents were brought to room temperature. The vial of Detection Antibody Cocktail which is lyophilized biotinylated antibodies was reconstituted in 100µl of deionized water. The bottle of 40ml of 25X Wash Buffer Concentrate was diluted into 960ml deionized water.

Two milliliters of Array Buffer 4 were pipetted into each well of the four-well multi-dish used. The membranes to be used were removed from between the protective sheets by flat-tip tweezers and placed in one well of the dish. The array number was kept facing upward. The membranes were incubated for 1 hr on a rocking platform to block non-specific bindings. One ml of the supernatant to be tested was taken out and added to separate tubes with 0.5ml of Array Buffer 4. Fifteen microliters of reconstituted Cytokine Array Panel Detection Antibody Cocktail was added to each prepared sample and mixed incubated at room temperature for 1 hr. After 1 hr of blocking, Array Buffer 4 was aspirated from the wells of the dish and prepared sample/antibody mixtures were added. The four-well multi-dish was then incubated overnight at 2-8 ℃ on a rocking platform with the lid on.

The next day, each membrane was removed and placed in individual plastic containers with 20ml of 1X Wash Buffer. The four-well multi-dish was rinsed with deionized water and dried thoroughly. Each membrane was washed three times on a rocking platform shaker, 10 minutes for each wash. The Streptavidin-HRP was diluted in Array Buffer 5 to a ratio of 1:2000, and 1.5ml of diluted Streptavidin-HRP was pipetted into each well of the four-well multi-dish. The dish was incubated for

30 minutes on a rocking platform with the lid on. After that, each membrane was washed three times more, and incubated with chemiluminescent detection reagent for two minutes. The binding was visualized with enhanced chemiluminescence (ECL) through Bio-RAD Image Lab™ and Bio-RAD software version 2.0. The quantification tool in the software enabled a relative reading to be taken for each spot and the average of each pair was considered as the reading for each cytokine.

### 3.11 Human Phospho-Kinase Array

To prepare the samples, eighty per cent confluent HMVEC-Ls cells were incubated with 1μg/ml galectin-3 or BSA control at 37℃ for 24 hr before release from the culture flasks by trypsinization and centrifuged at 1000 (**g*) for five minutes to remove the supernatant. The cell pellets were resuspended by 10ml PBS before the cell suspension was immediately transferred into two tubes pre-cooled on an ice box. After centrifugation at 1000 (**g*) for five minutes at 4℃, the tubes were put back on the ice box and the supernatants from the samples were completely removed. The cell pellet was lysed by 0.4ml/tube Lysis Buffer 6 from the Human Phosphorylation Kinase Array Kit, pipetted up and down several times and left on the rocking platform in the ice box for 30 minutes. Then each tube was centrifuged at 14000 x g for five minutes, before the supernatants were transferred into clean tubes and used as samples.

To prepare the array, 1.0ml of Array Buffer 1 was added to four wells of an eight-well multi-dish. Each membrane to be used was removed from between the

protective sheets and placed in each well with buffer. After 1 hr incubation on a rocking platform, Array Buffer 1 was removed and 334ul/each sample was added to 1666µL/each of Array Buffer1 and 1.0ml of each diluted sample was added to both Part A and Part B membranes. The plate was incubated overnight with a lid on at 2-8℃ on a rocking platform.

The next day, each membrane was removed and placed in individual plastic containers, and washed by 20ml of 1x Wash Buffer for 10 minutes on a rocking platform three times. For each Part A membrane, 20µL of reconstituted Detection Antibody Cocktail a was diluted to 1.0mL with 1X Array Buffer 2/3, and 1.0ml diluted Detection Antibody Cocktail A was added to the wells of an eight-well multi-dish. Each Part A membrane was returned to the wells containing the diluted Detection Antibody Cocktail A. For each Part B membrane, 20µL of reconstituted Detection Antibody Cocktail B was diluted to 1.0mL with 1X Array Buffer 2/3, and 1.0ml diluted Detection Antibody Cocktail A was added to the wells of an eight-well multi-dish. Each Part A membrane was returned to the wells containing the diluted Detection Antibody Cocktail B. After the plate was covered by the lid, it was placed on the rocking platform to incubate for two hours at room temperature. After that, each membrane was placed in individual plastic containers with 20ml of 1X Wash Buffer, and washed three times for ten minutes on a rocking platform. The Streptavidin-HRP was diluted in 1X Array Buffer 2/3 and 1.0 mL diluted Streptavidin-HRP was added and incubated for 30 minutes in each well of the eight-well multi-dish. After three more washes, the membranes were exposed to chemiluminescent reagents for 2

minutes and visualized with enhanced chemiluminescence (ECL) through Bio-RAD Image Lab™ and Bio-RAD software version 2.0.

### 3.12 Sandwich ELISA Assay for Cytokine quantification in Cell Culture Supernatants and Human Serum Samples

HMVECs were released from the culture flasks by trypsinization (1ml Trypsin for each T25 flask) and suspended at 1 x $10^5$/ml with EBM-2 culture medium. HMVEC suspensions were cultured in a 12-well plate (1ml/well) at 37℃ for 24 hr before introduction of a certain concentration of recombinant galectin-3 or BSA for 24 hr. The conditioned medium (CM) was collected and the concentrations of cytokines in the conditioned medium were analyzed using Cytokine ELISA kits (R&D systems)

Wells of a high-binding 96-well plate of half volume were coated with 50µl of Cytokine Capture Antibody (either anti-G-CSF: 2.0µg/ml; anti-GM-CSF: 2.0µg/ml; anti-IL-6: 2.0µg/ml; or anti-ICAM-1: 4.0µg/ml). The plate was sealed and left overnight at room temperature. On the next day, the plate was washed three times with 150µl per well washing buffer (0.05% Tween20 in PBS) and 150µl of Reagent Diluent (1% Bovine Serum Albumin (BSA) in PBS, pH 7.2-7.4) was applied to each well for a minimum of 1 hr as blocking buffer. The wells were washed another three times with 100µl washing buffer per well per time. To provide samples, the conditioned medium was collected and centrifuged at 1000 (**g*) for 1 min to remove floating cells, while the human serum samples were diluted with PBS. Then 6-9 points' serial twofold standard dilutions as well as 50µl of samples were added to the

wells and incubated for at least two hours. After that, the wells were washed three times with 100µl washing buffer per well and 50µl of detection antibody, were added to the wells (biotinylated anti-G-CSF: 200ng/ml; biotinylated anti-GM-CSF: 500ng/ml; biotinylated anti-IL-6: 50ng/ml; biotinylated anti-ICAM-1: 100ng/ml, diluted in reagent diluent) and incubated for at least two hours. The wells were washed three times with 100µl washing buffer per well per time, and 50µl of Streptavidin-HRP at working concentration (1:200) was added to each well and incubated for 20 minutes at room temperature. The wells were washed three times with 100µl washing buffer per well. Sigma-Aldrich FAST OPD tablets (Sigma-Aldrich) were dissolved in 20ml distilled $H_2O$ and 50µl applied to each well for approximately 20 minutes until the color turned from an yellow/orange color developed in the standard curve of the cytokine standard. The reaction was stopped with 25µl 4M sulphuric acid, concentration-associated visible light intensity was measured with a TECAN Sunrise microplate reader in which the subtract readings setting was 492nm.

## 3.13 *In vivo* experiments to assess effects of galectin-3 on cytokine secretion

In the study, balb/c nude female mice age 7-8 weeks were used. They were housed in specific pathogen free conditions with a 12:12 hour light: dark cycle with free access to food and water. The project license number is 40/3392.

To assess the influence of galectin-3 on cytokine secretion *in vivo,* we injected galectin-3, at concentrations designed to reproduce similar circulating

concentrations to those found in patients with metastasis, intravenously via the tail vein into the mice and 100µl blood was taken from three mice without galectin-3 injection to use as control. Then 100µl galectin-3 (5µg/mouse) was injected into three mice and after 24 hr, these three mice were sacrificed by $CO_2$ and intra-cardiac puncture was carried out. Then 5µg/mouse galectin-3 (100µl) was injected into another three mice and after 48 hr the mice were sacrificed by $CO_2$ and intra-cardiac puncture was carried out. The cytokine concentrations in the collected sera were later assessed by means of ELISA.

### 3.14 Sandwich ELISA Assay for the quantification of cytokines in Mouse Sera

Wells of a high-binding 96-well plate of half volume were coated with 50µl of Cytokine Capture Antibody (either anti-G-CSF: 0.5µg/ml; anti-GM-CSF: 0.5µg/ml or anti-IL-6: 2.0µg/ml). The plate was sealed and left overnight at room temperature. On the next day, the plate was washed three times with 150µl per well washing buffer (0.05% Tween20 in PBS) and 150µl of Reagent Diluent (1% BSA in PBS, pH 7.2-7.4) was applied to each well for a minimum of 1 hour as blocking buffer. The wells were washed another three times with 100µl washing buffer per well per time. Mouse serum samples were diluted by PBS after pilot studies to assure that the final concentration was in the measuring range of the kit. Then 6-9 points' serial twofold standard dilutions as well as 50µl of samples were added to the wells and incubated for at least two hours. After that, the wells were washed three times with 100µl

washing buffer per well. Then 50µl of detection antibody were added to the wells (either biotinylated anti-G-CSF: 250ng/ml; anti-GM-CSF: 250ng/ml or anti-IL-6: 500ng/ml) and incubated for at least two hours. The wells were washed three times with 100µl washing buffer per well and 50µl of Streptavidin-HRP at working concentration (1:2000) were added to each well and incubated for 20 minutes at room temperature. The wells were washed three times with 100µl washing buffer per well per time. Sigma-Aldrich FAST OPD tablets (Sigma-Aldrich) were dissolved in 20ml distilled $H_2O$ and 50µl applied to each well for approximately 20 minutes until the color turned from an yellow/orange color developed in the standard curve of the cytokine standard. The reaction was stopped with 25µl 4M sulphuric acid, concentration-associated visible light intensity was measured with a TECAN Sunrise microplate reader in which the subtract readings setting was 492nm.

## 3.15 Quantification of sICAM-1 in Mouse Sera by ELISA

Mouse sera obtained as above were diluted and the levels of sICAM-1 were assessed with Quantikine ELISA kits.

The Kit Control was reconstituted with 1.0mL distilled water. Then 25mL Wash Buffer concentrate was added to distilled water to obtain 625 mL of Wash Buffer. Then 20 mL of Calibrator Diluent RD5-26 Concentration was added to 60 mL of distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (1X). Mouse sICAM-1 Standard was reconstituted by adding 5.0 ml of Calibrator Diluent RD5-26 (1X). After

the standard had been thoroughly mixed it was kept for 5 minutes with gentle mixing prior to making dilutions.

Then 50µl of Assay Diluent RD1-21 was added to each well followed by 50µl of standard and samples, and incubated for 2 hours. Each well on the plate was washed three times with 400µl Wash Buffer per well five times. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Next 100µl of Mouse sICAM-1 Conjugate was added to each well followed by two hours' incubation. After another five washes, 100µl of Substrate Solution was added to each well and incubated for 30 minutes without exposure to the light. The reaction was stopped with 100µl Stop Solution from the kit and read by a plate microreader (Tecan), in which the subtract readings setting was OD 570nm from the reading at OD 450nm.

### 3.16 Assessment of Endothelial Cell Surface Adhesion Molecule expression by Flow Cytometry

Sub-confluent HMVEC-Ls cells were treated with galectin-3 (1.5µg/ml) in the presence or absence of 10µM lactose for 24 hr or recombinant G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml) as a group or separately for 1 hr before they were released by 1ml non-enzymatic cell dissociation solution (0.5mg/ml) incubated for 5-10 minutes at 37℃.The cells were washed with 10ml sterile PBS and the supernatant discarded after centrifugation at 1000 (**g*) for five minutes. After removal of the supernatant, 5ml 2% paraformaldehyde was added to

fix the cells for at least 25 minutes at room temperature. Then the cells were washed twice with 10ml PBS and centrifuged at 1000 (**g*) to remove the supernatants. The number of cells was counted and the cells were resuspended at a concentration of $10^6$/ml with PBS and incubated with 5% goat serum for 30 minutes at room temperature on the roller. After removal of the supernatant following centrifugation at 1000 (**g*) for 5 minutes, the cells were resuspended at the concentration of $10^6$/ml in the 1% goat serum in PBS and divided 1ml/tube in 1.5ml tubes. Antibodies against CD44 (1mg/ml), α5β1 (1mg/ml), α5β3 (1mg/ml), E-selectin (1mg/ml), VCAM (1mg/ml) and ICAM (1mg/ml) all in 1:400 dilution with PBS were applied to the cell solutions for 1 hr at room temperature on the roller (or overnight at 4℃). After they were washed twice with PBS, fluorescence (FITC)-conjugated secondary antibodies (1:400 in 1% BSA in PBS) were applied for 1 hour at room temperature. After three washes with PBS, the cells were resuspended in PBS in 0.5ml/tube. The cell surface expression of CD44, α5β1, α5β3, E-selectin, VCAM and ICAM was analyzed by flow cytometry. Fluorescent conjugated secondary antibody without addition of the primary antibody was used as a negative control in all the experiments.

### 3.17 *In Vitro* Angiogenesis Assay- Endothelial Cell Invasion

The HMVEC cells were released by trypsinization and were seeded into a 24-well plate for 24 hours at a concentration of 1 x $10^5$/ml in EBM-2 medium. Then to each pair of wells were added 1.5μg/ml BSA (control), recombinant galectins-3 or

recombinant galectins-3 + 100μM final concentration of lactose. The plate was incubated at 37℃ for 48 hr for production of the conditioned medium (CM).

To obtain an invasion device 5ml of 1X BME solution were made in a sterile 15ml conical tube and gently inverted to mix on ice and 50μl of BME Coat and then 50μl 1X Coating Buffer were added to the top chamber of each well of a 24-well cell invasion device. Dispersion of coating of wells can be visually inspected by gently tapping the side of the cell invasion chamber a few times. The cell invasion chamber was incubated overnight at 37℃ in a $CO_2$ incubator.

A fresh flask of HMVEC-Ls cells was harvested with Trypsin and washed with PBS. After being centrifuged at 1000 (**g*) for five minutes, the cells were resuspended at a concentration of 4 x $10^5$cells/ml with EBM-2 medium. The cell suspension was mixed and aliquoted to 200μl/each to sterile centrifuge tubes. The cells were then centrifuged at 1000 (**g*) for five minutes and resuspended with 200μl CMs collected from the 24-well plate of the HMVEC-Ls culture with and without addition of neutralizing anti-cytokine antibodies or recombinant cytokines (BSA-treated control group; Gal-3-treated group; Gal-3-plus 100μM lactose inhibitory group; Gal-3-treated group plus four anti-cytokine antibodies (G-CSF, GM-CSF, IL-6 and sICAM-1); BSA-treated control plus four recombinant cytokines (G-CSF, GM-CSF, IL-6 and sICAM-1)). Coating solution was carefully aspirated from the top chamber of the cell invasion device, and the top and bottom chambers were kept wet all the time. Then 50μl of cell suspension (20,000 cells /well) were added to each well of the top chamber and 150μl/well of serum-free EBM-2 medium were added to each well

of the bottom chamber. Then the cells were incubated at 37℃ in a $CO_2$ incubator for 24 hours.

After incubation, the top chamber was aspirated and washed with 100µl of 1X wash buffer without puncturing the membrane. The bottom chamber wells were also aspirated by disassembling and reassembling device with the assay chamber plate for minimal background signal. Then 10µl of Calcein-AM solution were mixed with 10ml of 1X Cell Dissociation Solution and 250µl/well of Cell Dissociation Solution/Calcein AM were added to the bottom chamber at 37℃ in the $CO_2$ incubator for 30 minutes. The plate was tapped 10 times on the side gently and incubated at 37℃ for an additional 30 minutes (1 hr in total). After disassembly of the cell migration device, the assay chamber (bottom) was read at 485nm excitation / 520nm emission.

### 3.18 *In Vitro* Angiogenesis Assay - Endothelial Tube Formation

Endothelial cells (HMVEC-Ls/HUVEC) were seeded in a T25 tissue culture flask until 80% confluence was reached prior to assay. Cultrex RGF BME was thawed at 2-4℃ overnight in a refrigerator. The tips and 96-well plates to be used were also kept in the refrigerator overnight.

50µl of BME solution were aliquoted into each well of a 96-well plate on ice. The plate was centrifuged at 1000 (**g*) for 5 minutes at 4℃ to make sure no air bubbles were trapped in the BME and then the plate was incubated at 37℃ for 45

minutes. Then 5µl of 2mM Calcein AM Working Solution were added to 5ml EBM-2 to make a 2µM Calcein AM solution. HMVEC-Ls/HUVEC cells were incubated with Calcein AM solution (5 ml per T25 flask) for 30 minutes at 37℃ in a $CO_2$ incubator. The cells were washed twice with 5ml sterile room temperature PBS and 1ml of Trypsin was added, before the cells were incubated at 37℃ for four minutes. After that, 10ml of PBS were added and the cells were transferred into a 15ml conical tube. The cells were centrifuged at 1000 (**g*) for four minutes, and the supernatant was removed. The cells were resuspended with 200µl PBS. The cells were diluted in CM collected from HMVECs treated with/without galectin with/without the presence of the inhibitors and with/without anti-cytokine antibodies or recombinant cytokines as above. Then 100µl of diluted cells (1 x $10^5$ cells /ml) were added slowly to each well containing gelled BME. The plate was then incubated at 37℃ in a $CO_2$ incubator for three to 24 hours. The tube formations were visualized by microscope. The pictures of the whole wells of each group were taken and the number of tubes and nodules as well as the length of each tube within were measured and recorded.

### 3.19 Human Matrix metalloproteinase Array

HMVEC-Ls were released from the culture flasks by trypsinization (1ml Trypsin for each T25 flask) and suspended at 2 x $10^5$/ml with EBM-2 culture medium before the application of 200µl/well to each well on the top chamber of the cell invasion device while 150µl/well serum-free EBM-2 were added to each well on the bottom chamber before incubation for 24 hr at 37℃. The medium in the top

chamber wells was replaced with fresh EGM-2 medium after three hours to remove residual Trypsin. On the next day, the medium in the top chamber wells was replaced by a medium with 1.5μg/ml BSA (control) or recombinant galectins-3 respectively, and the serum-free EBM-2 was renewed as well. After 24 hr incubation at 37℃, the medium in the bottom chamber wells was collected and the levels of MMP were analyzed.

Each membrane in the array was placed into the provided eight-well tray before 2 ml 2X Blocking Buffer were added and incubated at room temperature for 30 minutes. After Blocking Buffer was decanted, 1 ml of collected conditioned medium was added to each well to incubate with the membrane overnight at 4℃. After the conditioned medium was discarded from each well, each membrane was washed three times (each for 5 minutes) with 2 ml of 1X Wash Buffer I at room temperature with shaking, followed by two washes (each for 5 minutes) with 2 ml of 1X Wash Buffer II at room temperature with shaking. Then 1 ml of diluted biotin-conjugated antibodies was added to each membrane and incubated at room temperature for two hours. After each well was decanted, each membrane in the well was washed three times (each for 5 minutes) with 2 ml of 1X Wash Buffer I at room temperature with shaking, followed by two washes (each for 5 minutes) with 2 ml of 1X Wash Buffer II at room temperature with shaking. Then 2 ml of diluted HRP-Conjugated Streptavidin were added to each well and incubated for two hours. Each well was decanted, and each membrane in the well was washed three times (each for 5 minutes) with 2 ml of 1X Wash Buffer I at room temperature with shaking, followed by two washes (each for 5 minutes) with 2 ml of 1X Wash Buffer II at room

temperature with shaking. For each membrane, 250µl of 1X Detection Buffer C and 250µl of 1X Detection Buffer D was mixed, which was pipetted onto the membrane on a clean plastic sheet provided by the kit and incubated for 2 minutes. After the detection reagent was drained off, another piece of plastic sheet was placed on the array and the chemiluminescence signal was visualized with Bio-RAD Image Lab™ and Bio-RAD software version 2.0.

### 3.20 *In Vivo* Assessment of the effect of Galectin-3 Induced Cytokine Secretion on Metastasis in Mice

To directly assess the influence of galectin-3-induced cytokine secretion on metastasis, either PBS or a combination of cytokines (2µg ICAM-1 and 20ng each of GM-CSF, G-CSF and IL-6) was injected at similar levels to those observed in mouse serum following galectin-3 injection. One dose was administered 4 hr prior to injection of ACA19$^{-}$ cells mixed with a second dose of PBS or cytokines. Eight mice were treated per experimental group. One mouse per group was sacrificed after four weeks and six weeks post-injection and metastatic foci in the lungs were examined by direct observation through a microscope and by section staining. Remaining animals were scarified eight weeks post-injection in order to develop macroscopic tumors, and metastases on the lung, liver, brain and kidney were all examined (this part of the experiment was conducted by Dr Carrie Duckworth in our department).

## 3.21 Human Samples

Fifty serum samples from colorectal cancer patients (aged 25 to 91), 39 without clinically detectable metastasis (25 males and 14 females) and 11 with liver metastasis (seven males and four females) were obtained from the Cancer Tissue Bank Research Centre (CTBRC) cancer tissue bank (Liverpool, UK). These serum samples were obtained from patients at the time of primary tumor resection at the Royal Liverpool University Hospital. The length of survival of these patients in the next 10 years was followed up. Thirty-one serum samples from healthy people (13 Male and 19 female) were obtained from Sera Laboratories International (Haywards Heath, UK). The patients' ages were from 25 to 91 (mean age 63).

## 3.22 Statistics

Data were analyzed using independent t test, analysis of variance and one-way ANOVA followed by Bonferroni or Spearman rank correlation depending on the experiment or context. Two-sided p-values with values $< 0.05$ were reported as statistically significant.

# CHAPTER 4 INVESTIGATION OF THE MUC1-INDEPENDENT ACTION OF CIRCULATING GALECTIN-3 ON METASTASIS PROMOTION

## 4.1 Hypothesis and Aim

### 4.1.1 Hypothesis

Circulating galectin-3 has MUC1-independent actions in its effect on metastasis promotion that may be mediated via cytokine release and its subsequent impact on cell-endothelial adhesion.

### 4.1.2 Aim

To investigate the MUC1-independent actions of circulating galectin-3 on metastasis promotion, including effects on cytokine production and cell-endothelial adhesion.

## 4.2 INTRODUCTION

Galectin-3 is a galactoside-binding, multi-functional protein that is expressed by many types of human cells. Galectin-3 is found inside cells, extracellularly on the cell surface as well as in the blood circulation. Intracellular galectin-3 is an apoptosis inhibitor and mRNA splicing promoter (384) whereas cell surface-associated

extracellular galectin-3 acts as an adhesion molecule in cell-cell and cell-matrix interactions and facilitates cancer progression and metastasis(385). The concentrations of free circulating galectin-3 are increased up to 30-fold in the bloodstream of various cancer patients including breast, colorectal(321), lung(322), bladder(323), head and neck(324) and melanoma(325). Patients with metastatic disease are also seen to have higher concentrations of circulating galectin-3 than those with only localized tumors. In patients with melanoma, a high serum galectin-3 level has been shown to correlate significantly with poor outcome (386).

Our recent studies have shown that galectin-3 binds to the TF antigen on cancer-associated MUC1 and induces MUC1 cell surface polarization and exposure of the underlying adhesion molecules, which results in increased cancer cell adhesion to the vascular endothelium and increased cancer cell homotypic aggregation for the formation of tumor emboli in the circulation. Co-injection of galectin-3 with MUC1-positive human melanoma $ACA19^{+}$ cells to the tail vein of nude mice caused increased lung metastasis (326). These results suggest an important role of the increased circulation of galectin-3 in promoting tumor cell metastatic spread. Interestingly, co-injection of galectin-3 with the MUC1-negative human melanoma $ACA19^{-}$ cells also caused a smaller increase of lung metastasis of the animals (327). This indicated that circulating galectin-3 probably has another MUC1-independent action in its promotion of metastasis.

## 4.3 METHODS

### 4.3.1 Collection of conditioned medium from cancer cells

ACA19$^-$ and HCT116 cancer cells were detached from the culture plates by trypsinization and suspended at 2 x $10^5$/ml with serum-free DMEM medium before application in six-well plates in which every well to be used was pre-coated with Poly-HEMA. The cells were incubated for 24 hr at 37 ℃ before 1μg/ml galectin-3 or BSA was added to each well. After 1 hr or 24 hr treatment, the conditioned medium from each well was collected.

### 4.3.2 Collection of conditioned medium from HMVEC-Ls

As regards the HMVEC-Ls cells used for obtaining the conditioned medium, HMVEC-Ls were released from the culture flasks by trypsinization and suspended with EBM-2 culture medium at concentration of 2 x $10^5$/ml before application (2ml/well) to each well to be used in six-well plates. The medium in each well was replaced with fresh EGM-2 medium after three hours. The cells were incubated (one day) until the formation of cell monolayers before they were treated with 1μg/ml of galectin-3 or BSA in the presence or absence of lactose (10μM) for 1 hr or 24 hr. The conditioned medium from each well was collected for future use.

### 4.3.3 Assessment of galectin-3-mediated cancer cell-endothelial adhesion

HMVEC-Ls were released from the culture flasks by trypsinization and suspended with EBM-2 culture medium before application of 200µl/well to each well to be used in 96-well plates. The medium in each well was replaced with 150µl fresh EGM-2 medium after three hours. The cells were incubated at 37 ℃ until the formation of cell monolayers (one day) before they were treated with 1µg/ml of galectin-3 or BSA in the presence or absence of lactose (10µM) for 1 hr or 24 hr. The HMVEC monolayer was then washed and used for subsequent assessment of cancer cell adhesion.

HMVEC-Ls were released from the culture flasks by trypsinization and suspended at 2 x $10^5$/ml with EBM-2 culture medium before application of 200µl/well to each well to be used in 96-well plates. The medium in each well was replaced with 150µl fresh EGM-2 medium after three hours for one day for the formation of cell monolayers (one day). The HMVEC monolayer was then washed and used for subsequent assessment of cancer cell adhesion.

The ACA19$^-$ and HCT116 cancer cells used for assessment of cell adhesion were detached from the culture plates with NECDS, washed and resuspended in serum-free DMEM medium (for ACA19 or McCoy's 5a medium for HCT116). The ACA19$^-$ and HCT116 cancer cells were labeled with Calcein AM Cell Labeling Solution (10 µl for 1 ml of cell suspension). The ACA19$^-$ and HCT116 cancer cells were washed and resuspended with serum-free medium at concentration of 5 x $10^4$/ml before

application 100µl/well to HMVEC monolayer for 1 hr. The HMVEC monolayer was washed and the endothelial cell-associated fluorescence was measured.

### 4.3.4 Cytokine Determination by Human cytokine array

HMVEC-Ls were released from the culture flasks by trypsinization (1ml Trypsin for each T25 flask) and suspended at 1 x $10^5$/ml with EBM-2 culture medium. HMVEC-Ls suspensions were cultured in a six-well plate (2ml/well) at $37^0$C for 24 hr before the introduction of 1.0µg/ml recombinant galectin-3 or BSA for 24 hr. The conditioned media were collected and the concentrations of cytokines in the culture media were analyzed by Human Cytokine Protein Arrays.

For the assessment of galectin-3 effect on cytokine secretion from cancer cells, ACA19$^-$ cells were released from the culture flasks by trypsinization (1ml Trypsin for each T25 flask) and were seeded at a concentration of 5x$10^5$cells/ml into six-well plates that were pre-coated with 10mg/ml poly-HEMA for 24 hr (381). The cells were cultured under suspension condition in the presence of 1.0µg/ml galectin-3 or BSA for 24 hr at 37℃. The culture media were collected by 1000 (**g*) centrifugation of the cell suspension and the concentrations of cytokines in the culture media were analyzed by Human Cytokine Protein Arrays.

Briefly, 1ml of the conditioned medium was added to a separate tube with 15µl reconstituted Cytokine Array Panel Detection Antibody Cocktail. At the same time, the kit membranes to be used were incubated with blocking buffer for 1 hr to block

non-specific bindings. After removal of the blocking buffer by aspiration, the conditioned medium/detection antibody mixtures prepared were applied to the membrane for overnight incubation. The next day, each membrane was removed and washed three times for 10 minutes each time. Then 1.5µl diluted Streptavidin-HRP was incubated with membrane for 30 minutes followed by another three washes and two minutes' incubation with chemiluminescent detection reagent. The arrays were then visualized with the chemiluminescence testing device Bio-RAD Image Lab™.

## 4.4 RESULTS

### 4.4.1 Investigation of effect of long-term treatment of cancer cells with galectin-3 on their behavior in subsequent cancer cell-endothelial adhesion

Earlier studies in our lab have shown that galectin-3-MUC1 interaction-associated cancer cell adhesion occurs rapidly, within an hour (381, 387, 388). In searching for the MUC1-independent action of circulating galectin-3, we investigated whether the long-term presence of galectin-3 (24 hr) has any influence on the behaviors of MUC1-negative cancer cells. Cancer cells (ACA19$^-$ and HCT116) cultured in suspension at a concentration of 1 x $10^6$/ml were treated with 1µg/ml galectin-3, BSA or TNF-α (10µg/ml) (positive control) for 24 hr. After washing with PBS, labeling with Calcein AM, and further washing with PBS, the cancer cells were centrifuged for five minutes. The cell pellets were resuspended at a concentration of 1 x $10^5$/ml and

100µl of the suspension was added to the HMVEC-Ls monolayer for 1 hr. After two washes with PBS to remove the non-adhesion cells, the fluorescent intensity in each well was determined.

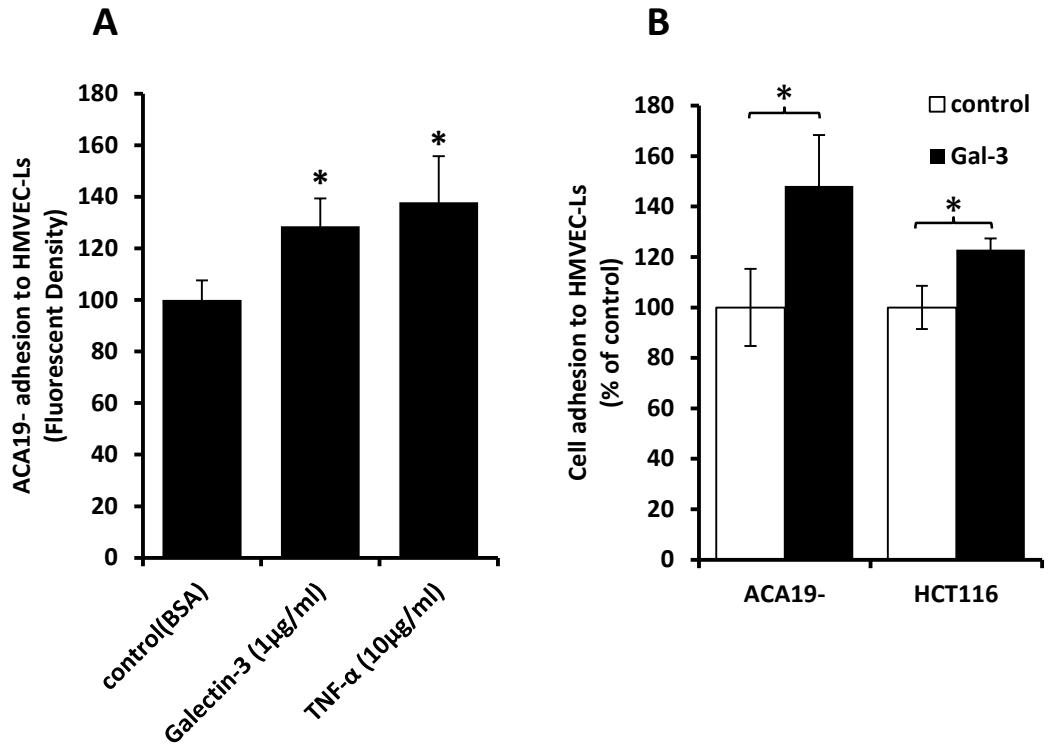


**Fig. 4.1: Long-term presence (24 hr) of galectin-3 increases MUC1-negative cancer cell adhesion to HMVEC-Ls.** (A) The ACA19$^-$ cells were treated with 1.0 µg/ml galectin-3, BSA or 10µg/ml TNF-α for 24 hr under suspension culture before 1 hr adhesion to HMVEC-Ls monolayer was assessed. (B) The ACA19$^-$ or HCT116 cells were treated with 1.0 µg/ml galectin-3 or BSA for 24 hr under suspension culture before 1 hr adhesion to HMVEC-Ls monolayer was assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05 (one-way ANOVA followed by Bonferroni)

It was found that, the presence of TNFα for 24 hr in the culture as a positive control showed an increase in ACA19- cell adhesion (Fig. 4.1 A). The presence of galectin-3 at a concentration (1μg/ml) similar to that seen in the blood circulation of cancer patients for 24 hr in the culture also resulted in significant increase of adhesion of ACA19- (48.2±8.5%) and HCA116 (22.9±4.4%) to HMVEC-Ls (Fig. 4.1 B).

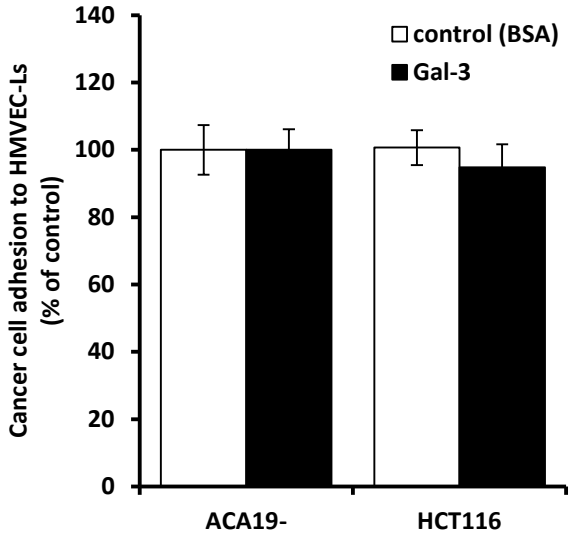


**Fig. 4.2: Short-term (1 hr) presence of galectin-3 does not influence MUC1-negative cancer cell adhesion to HMVEC-Ls.** The ACA19$^-$ or HCT116 cells were treated with 1μg/ml galectin-3 or BSA for 1 hr under suspension culture before 1 hr adhesion to HMVEC-Ls monolayer was assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments (unpaired t test).

Short-term (1 hr) presence of galectin-3 at 1.0 μg/ml did not seem, however, to have any significant influence on adhesion of MUC1-negative ACA19- and HCT116 cells to HMVEC-Ls (Fig. 4.2).

### 4.4.2 Investigation of effect of the conditioned medium obtained from galectin-3-treated cancer cells on subsequent cancer cell adhesion to HMVEC-Ls

It was found that the culture medium (CM) obtained from 24 hr galectin-3 (1μg/ml) treated ACA19- or HCT116 cells had no effect on subsequent adhesion of fresh ACA19- or HCT116 cells to fresh HMVEC-Ls.

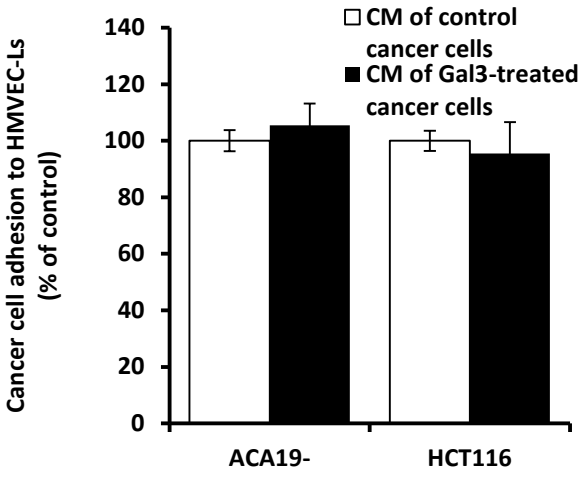


**Fig. 4.3: Galectin-3 does not induce secretion of soluble molecules from cancer cells that cause cancer cell-endothelial adhesion.** The 24-hr conditioned medium (CM) obtained from ACA19$^-$ or HCT116 cells treated with or without 1μg/ml galectin-3 under suspension condition were used as culture medium to assess adhesion of fresh ACA19$^-$ or HCT116 to the fresh HMVEC-Ls monolayer. The data are expressed as mean ± SD of triplicate determinations of three independent experiments (unpaired t test).

This indicates that long-term treatment of galectin-3-mediated cancer cell adhesion is not related to cancer cell secretion of soluble factors (Fig. 4.3).

### 4.4.3 Investigation of effect of long- or short-term treatment of HMVEC-Ls with galectin-3 on their behavior in subsequent cancer cell-endothelial adhesion

HMVEC-Ls monolayers were treated with 1.0 μg/ml galectin-3 for 1 hr/24 hr before washed by PBS. Fresh cancer cells (ACA19$^-$ or HCT116) were detached from the culture plates with NECDS and washed before being labeled with Calcein AM for half an hour. The cancer cells were washed again by PBS before being resuspended at a concentration of 1 x $10^5$/ml and 100μl cell suspension was added to each well to adhere to the galectin-3-pretreated HMVEC-Ls monolayer for 1 hr. After two washes with PBS, the fluorescent intensity of each well was determined.

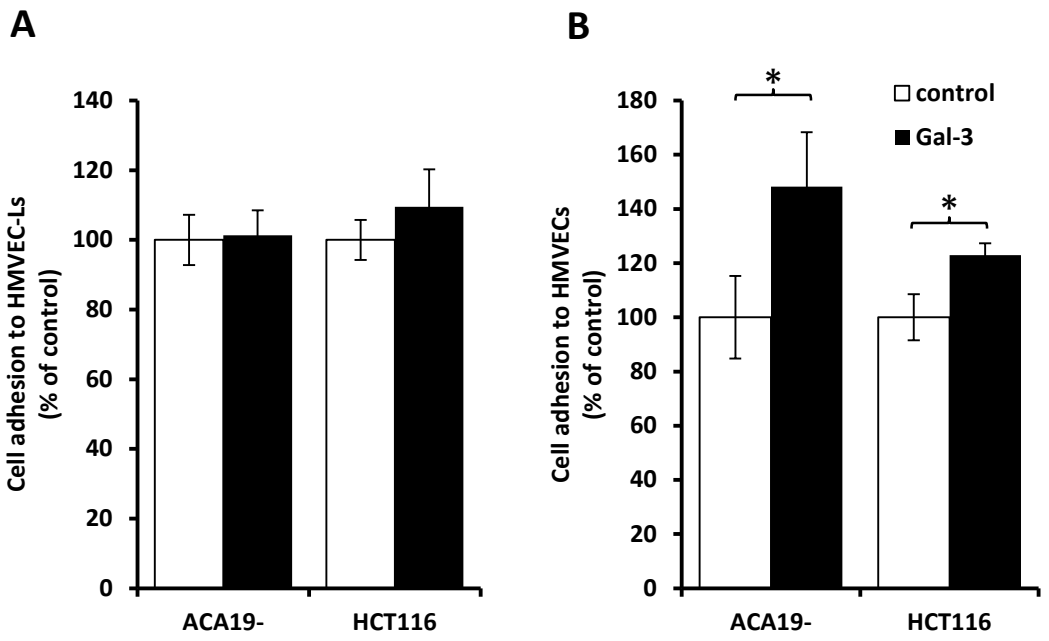


**Fig. 4.4: The long-term (24 hr) but not the short-term (1 hr) presence of galectin-3 in HMVEC-Ls increases subsequent cancer cell-endothelium adhesion.** The HMVEC-Ls monolayer was treated with 1μg/ml galectin-3 for 1 hr/24 hr before 1 hr adhesion was assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05 (unpaired t test)

Pretreatment of the HMVEC-Ls with galectin-3 for 24 hr (Fig. 4.4 B), but not 1 hr (Fig. 4.4 A) at (1μg/ml) similar caused a significant increase (48.2±8.5% and 22.9±4.4%, respectively) in ACA19- and HCT116 cell adhesion.

### 4.4.4 Investigation of the effect of the conditioned medium obtained from galectin-3-treated HMVEC-Ls in cancer cell-endothelial adhesion

The conditioned medium obtained from 24 hr galectin-3 (1μg/ml) treated HMVEC-LS cells was collected and used as culture medium to assess adhesion of ACA19$^-$ or HCT116 to fresh HMVEC-Ls.

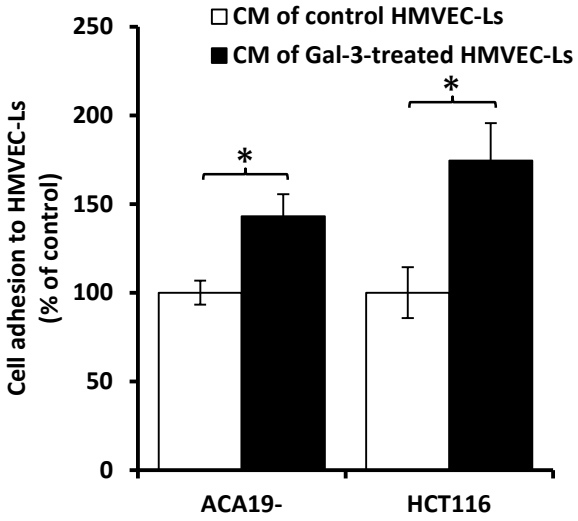


**Fig. 4.5: Galectin-3 induces secretion of soluble molecules from endothelial cells that cause cancer cell-endothelial adhesion in 1 hr.** The 24 hr-conditioned medium (CM) from HMVEC cells treated with or without 1μg/ml galectin-3 was used as culture medium to assess adhesion of fresh ACA19$^-$ or HCT116 to fresh HMVEC-Ls monolayer. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *P<0.05 (unpaired t test)

The conditioned medium obtained from HMVEC-Ls cells treated with galectin-3 (1μg/ml) for 24 hr appeared to induce a similarly increased adhesion of fresh ACA19$^-$ (43.0±14.3%) and HCT116 (74.4±21.2%) cells to fresh HMVEC-Ls (Fig. 4.5). This result

indicated that the presence of galectin-3 for a longer period induced release of soluble molecules from HMVEC cells, and this release by the endothelial cells is largely responsible for galectin-3-mediated adhesion of MUC1-negative cells to endothelial cells.

### 4.4.5 Investigation of effect of galectin-3 treatment with HMVEC-Ls or ACA19$^-$ cells on cytokine(s) secretion

Knowing that inflammatory cytokines such as TNFα, IL-1 and IL-6 are important metastasis-promoters and are known to promote several steps of the metastatic cascade including cancer cell adhesion to endothelium (389, 390), we investigated whether the soluble molecule(s) secreted by HMVEC-Ls in response to galectin-3 and responsible for galectin-3-mediated adhesion of MUC1-negative cells includes cytokine(s). We compared the cytokine profiles in the culture media of HMVEC-Ls and ACA19$^-$ cells treated with and without galectin-3 using a human cytokine array that covers 36 of the most common human cytokines.

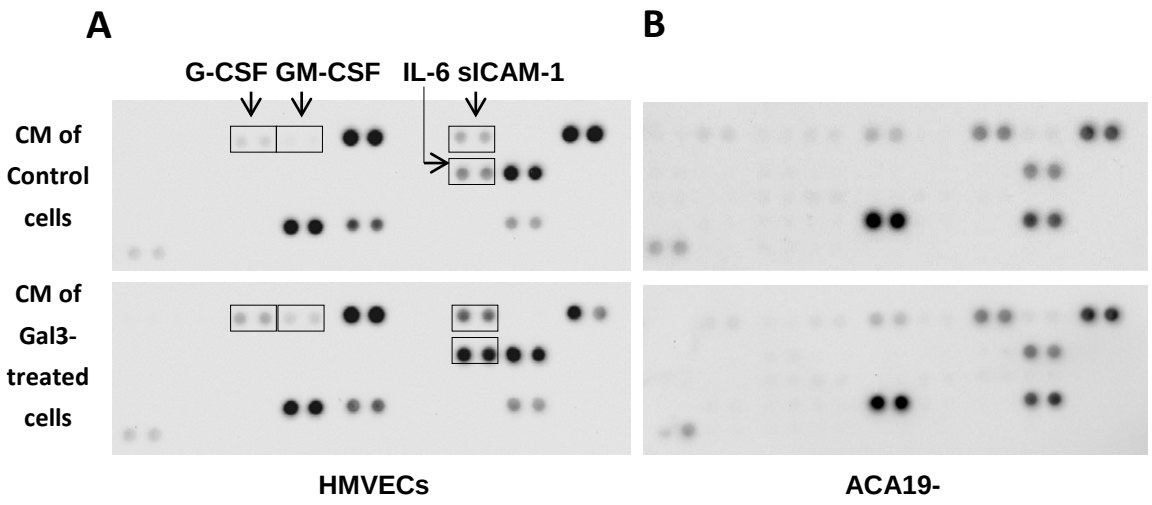


**Fig. 4.6: The presence of galectin-3 induces secretion of four cytokines secreted from HMVEC-Ls (A) but not ACA19$^-$ cells (B).** The levels of 36 common cytokines in the conditioned medium (CM) from HMVEC-Ls (A) cells treated with 1.0 µg/ml galectin-3 or BSA for 24 hr or from ACA19$^-$ cell (B) under suspension were assessed by cytokine protein array. Four cytokines (IL-6, G-CSF, GM-CSF and sICAM-1) showed more than twofold increase in the CM of galectin-3-treated HMVEC-Ls. The graph shown is representative of 2 independent experiments.

It was found that treatment of HMVEC-Ls with galectin-3 (1µg/ml) for 24 hr resulted in increased concentrations of four cytokines - IL-6 (2.1-fold), G-CSF (2.2-fold), GM-CSF (3.2-fold) and sICAM-1 (2.3-fold) - in the culture medium (Fig. 4.6 A) whereas galectin-3 treatment of ACA19- cells showed no difference in the cytokines in the culture medium (Fig. 4.6 B). This suggests that the presence of galectin-3 enhances cytokine secretion from HMVEC-Ls but not ACA19$^-$ cells.

### 4.4.6 Investigation of galectin-3-induced cytokine secretion from endothelial cells

#### 4.4.6.1 Investigation of effect of freeze-thaw cycle on galectin-3-induced cytokine secretion

We tested the effect of the freezing-thawing cycle of the conditioned medium obtained from galectin-3-treated HMVECs on the sensitivity of detection of these cytokines.

HMVEC-Ls cells were treated after 24 hr with various concentrations of galectin-3. The culture media were collected and separated into two groups. One group of samples was first frozen at -80℃ overnight before the levels of cytokines were tested and the other groups of samples were tested immediately.

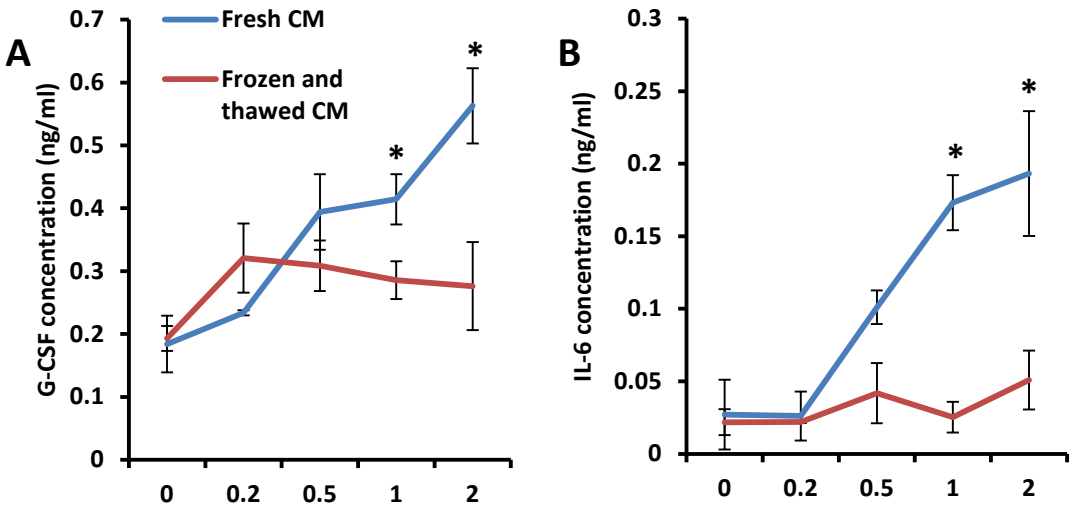


**Fig. 4.7: Galectin-3-induced secretion of G-CSF and IL-6 in the conditioned medium could not be detected after the samples were frozen and thawed.** HMVEC-Ls were treated with various galectin-3 concentrations for 24 hr before the concentrations of G-CSF and IL-6 in the culture media were determined. n=3. *p<0.05 (one-way ANOVA followed by Bonferroni).

It was found that the freezing and thawing cycle completely diminished the detection of these cytokines in the culture medium (Fig. 4.7), suggesting that the cytokines in the culture medium are sensitive to the freezing and thawing cycle. All the subsequent analyses were therefore conducted with fresh conditioned medium.

#### 4.4.6.2 Investigation of the dose-dependency of the galectin-3 effect on cytokine secretion

To investigate whether the G-CSF, GM-CSF, IL-6 and sICAM-1 secretions induced by galectin-3 were dose-dependent, confluent HMVEC-Ls cell monolayers were treated with galectin-3 in different concentrations (BSA control, 0.1, 0.2, 0.5, 1 and 2μg/ml) for 24 hr. Then the levels of G-CSF, GM-CSF, IL-6 and sICAM-1 in the conditioned medium were determined by individual cytokine ELISA.

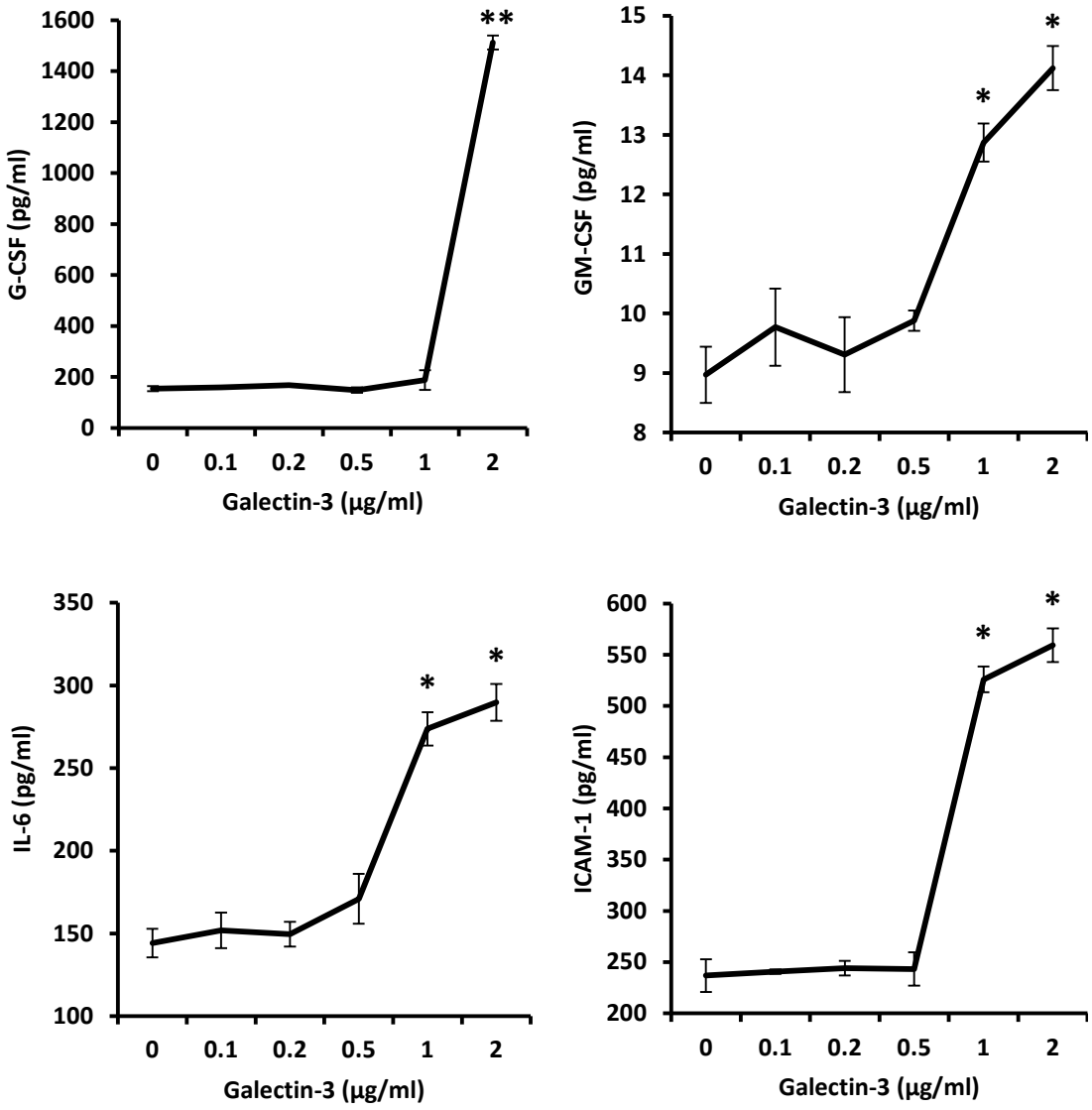


**Fig. 4.8: Galectin-3 induces dose-dependent secretion of G-CSF, GM-CSF, IL-6 and sICAM-1 from endothelial cells.** HMVEC-Ls were treated with or without various pathological galectin-3 concentrations (BSA control, 0.1, 0.2, 0.5, 1 and 2µg/ml) for 24 hr before the concentrations of each cytokine in the culture media were determined. n=3. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni).

It was found that the galectin-3-mediated cytokine secretion from HMVEC-Ls was galectin-3 dose-dependent and this induction occurred at galectin-3 concentrations similar to those found in the blood circulation of cancer patients (Fig. 4.8 and Table 4.1). At 2.0 μg /ml, galectin-3 induced increase of secretion of G-CSF, GM-CSF, IL-6 and sICAM-1 by 981.0±17.6%, 157.4±4.1%, 201.2±7.8% and 236.1±6.9%, respectively).

**Table 4.1 Galectin-3 induces dose-dependent secretion of G-CSF, GM-CSF, IL-6 and sICAM-1 from endothelial cells (n=3)**

| | Concentration (μg/ml) | Increase compare to dose 0 (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **G-CSF** | 0.1 | 103.5 | 2.96 | 0.595 |
| | 0.2 | 108.7 | 2.52 | 0.437 |
| | 0.5 | 96.2 | 6.76 | 0.354 |
| | 1 | 121.9 | 24.80 | 0.167 |
| | 2 | 980.9 | 17.63 | 0.005 |
| **GM-CSF** | 0.1 | 108.9 | 7.24 | 0.169 |
| | 0.2 | 103.8 | 7.02 | 0.243 |
| | 0.5 | 110.1 | 1.89 | 0.156 |
| | 1 | 143.5 | 3.57 | 0.034 |
| | 2 | 157.4 | 4.12 | 0.018 |
| **IL-6** | 0.1 | 105.3 | 7.35 | 0.506 |
| | 0.2 | 103.8 | 5.10 | 0.344 |
| | 0.5 | 118.5 | 10.50 | 0.387 |
| | 1 | 189.9 | 7.05 | 0.029 |
| | 2 | 201.1 | 7.80 | 0.026 |
| **ICAM-1** | 0.1 | 101.7 | 0.89 | 0.419 |
| | 0.2 | 103.0 | 3.02 | 0.423 |
| | 0.5 | 102.7 | 6.92 | 0.678 |
| | 1 | 222.0 | 5.28 | 0.021 |
| | 2 | 236.1 | 6.92 | 0.017 |

### 4.4.6.3 Investigation of the time-response of the galectin-3 effects on cytokine secretion from endothelial cells

A confluent HMVEC-Ls cell monolayer in a 96-well plate was treated with galectin-3 at 1µg/ml for different times (0, 4, 8, 10, 12, 14, 16, 18, 20, 24 and 48 hr) before the levels of G-CSF, GM-CSF, IL-6 and sICAM-1 in the conditioned medium were determined by individual cytokine ELISA.

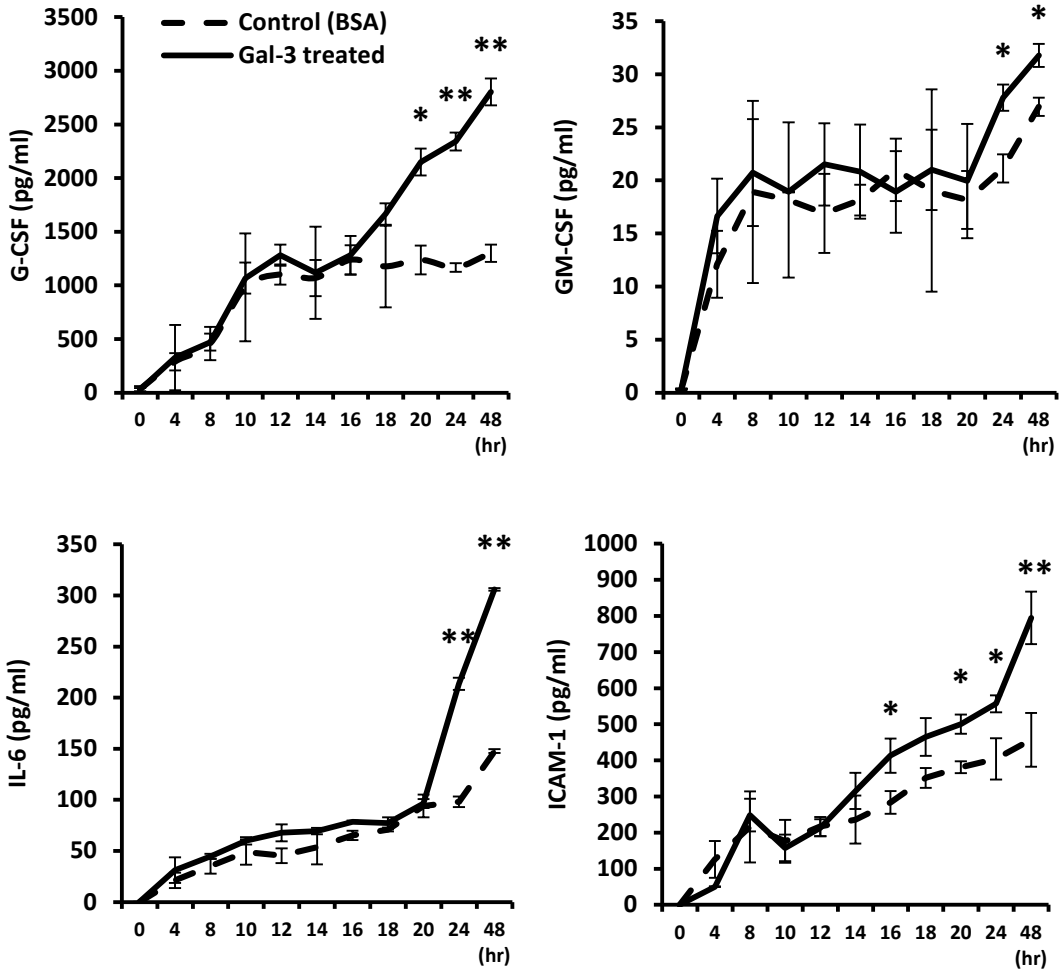


**Fig. 4.9: Galectin-3 induces time-dependent secretion of G-CSF, GM-CSF, IL-6 and ICAM-1 from endothelial cells.** HMVEC-Ls were treated with 1.5µg/ml galectin-3 or BSA for various time

points before the concentrations of G-CSF in the conditioned medium were determined. n=3. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni).

In comparison with the control BSA-treated cells, galectin-3 at 1.5ug/ml induced 115.7±9.7%, 17.9±3.2%, 107.1±1.3% and 73.9±16.3%, respectively (Fig. 4.9 and Table 4.2), increasing secretion of G-CSF, GM-CSF, IL-6 and sICAM-1 from HMVECs after 24 hr.

**Table 4.2 Galectin-3 induces time-dependent secretion of G-CSF, GM-CSF, IL-6 and ICAM-1 from endothelial cells (n=3)**

| | Time point (hr) | Increase compare to BSA treated control (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **G-CSF** | 18 | 41.6 | 8.50 | 0.2157 |
| | 20 | 73.6 | 10.12 | 0.0178 |
| | 24 | 100.3 | 7.12 | 0.0025 |
| | 48 | 115.7 | 9.71 | 0.0011 |
| **GM-CSF** | 20 | 9.9 | 15.04 | 0.3257 |
| | 24 | 31.4 | 6.24 | 0.0247 |
| | 48 | 17.9 | 3.15 | 0.0350 |
| **IL-6** | 20 | 2.61 | 11.86 | 0.7245 |
| | 24 | 117.7 | 5.43 | 0.0028 |
| | 48 | 107.1 | 1.25 | 0.0018 |
| **ICAM-1** | 14 | 33.6 | 28.12 | 0.2384 |
| | 16 | 45.7 | 11.07 | 0.0485 |
| | 18 | 32.1 | 7.78 | 0.0853 |
| | 20 | 31.4 | 4.31 | 0.03211 |
| | 24 | 37.8 | 14.19 | 0.03119 |
| | 48 | 73.9 | 16.26 | 0.00402 |

### 4.4.7 Investigation of effect of lactose on galectin-mediated cytokine secretion from HMVEC-Ls and on cancer cell adhesion

To further confirm the effect of galectin-3 on secretion of cytokines by HMVEC-Ls, we assessed the effect of the presence of the galectin-binding inhibitor lactose on galectin-mediated cytokine secretion.

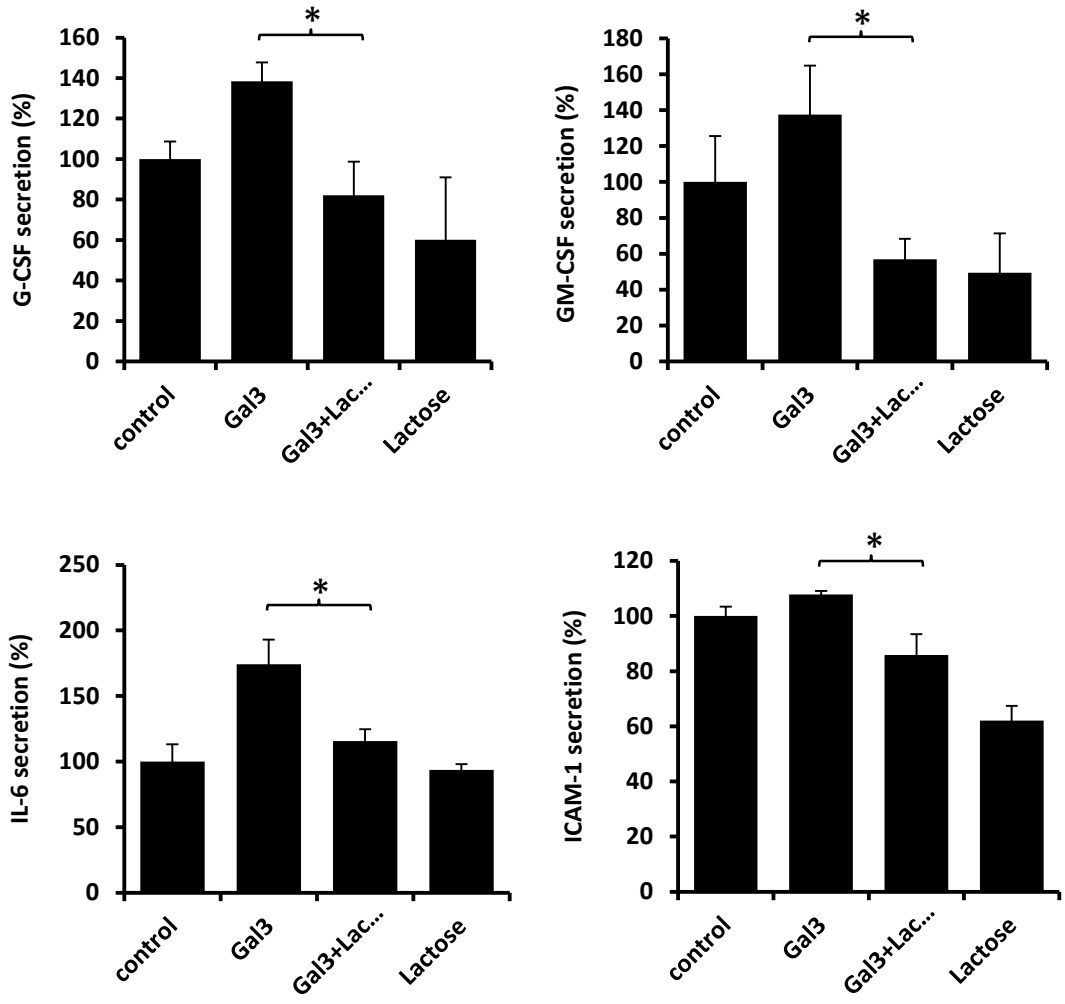


**Fig. 4.10 Galectin-3-mediated cytokine secretion can be inhibited by the presence of lactose.** HMVEC-Ls were treated with 1µg/ml BSA or galectin-3 in the presence or absence of 10µM lactose for 24 hrs before the cytokine levels in the conditioned medium were determined.

The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05 (one-way ANOVA followed by Bonferroni).

It was found that the increased secretion of G-CSF, GM-CSF and sICAM-1 induced by galectin-3(38.4 ± 9.4%, 37.4 ± 27.4% and 7.8 ± 1.2%, respectively) was abolished by the presence of lactose, whereas the increased secretion of IL-6 induced by galectin-3(74.2 ± 18.9%) was largely inhibited (84.4 ± 12.0%) in the presence of lactose (Fig. 4.10).

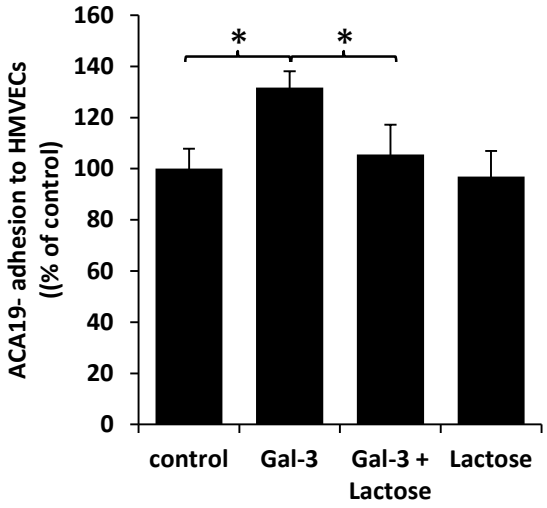


**Fig. 4.11 Lactose abolishes galectin-3-induced cancer cell-endothelial adhesion.**

HMVEC-Ls cells were treated with or without 1.5µg/ml galectin-3 in the presence or absence of 100µM lactose for 24 hr before adhesion of the fresh cancer cells to HMVEC-Ls were assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05 (One-way ANOVA followed by Bonferroni)

The presence of lactose also proved to effectively inhibit galectin-3-mediated ACA19$^-$ cell adhesion to HMVEC-Ls (Fig. 4.11). These results indicate that galectin-3-mediated cytokine secretion by endothelial cells and subsequent increased adhesion were mediated through the galectin-3 carbohydrate-binding sites.

### 4.4.8 Investigation of effect of a combination of anti-cytokine antibodies on galectin-3-mediated cancer cell-endothelial adhesion

To determine whether the galectin-3-induced secretion of IL-6, G-CSF, GM-CSF and sICAM-1 from HMVEC-Ls was responsible for galectin-3-mediated MUC1-negative cells adhesion to endothelium, we assessed the effect of antibodies against IL-6, G-CSF, GM-CSF and sICAM-1 on galectin-3-mediated ACA19- cell adhesion to HMVEC-Ls.

Confluent HMVEC-Ls cell monolayers in each well in a 96-well plate were treated with galectin-3 (1.5µg/ml) for 24 hr and BSA was used as negative control. After 24 hr incubation, the medium in wells were collected. An anti-cytokine antibody mixture [anti-G-CSF (50ng/ml), anti-GM-CSF (600pg/ml), anti-IL-6(4ng/ml) and anti-ICAM-1 (10ng/ml)] were added to the conditioned medium as an additional group. Fresh ACA19$^-$ cells were cultured with the collected medium or the collected medium with anti-cytokine antibodies mixture for an hour, and then the treated ACA19$^-$ suspension at a concentration of 1 x $10^5$/ml was added (100µl/well) to each well of the fresh HMVEC-Ls monolayer for 1 hr.

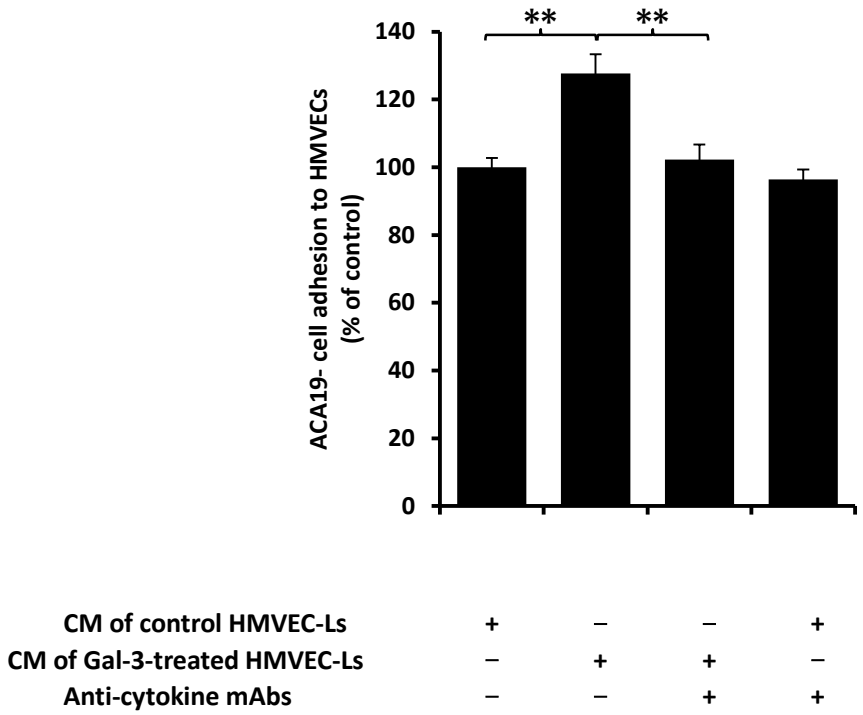


**Fig. 4.12 Galectin-3-mediated cancer cell-endothelial adhesion can be inhibited by the presence of anti-cytokine neutralizing antibodies.** The HMVEC monolayer was treated with 1.5μg/ml galectin-3 or BSA for 24 hr before the conditioned medium was obtained and fresh ACA19$^-$ was treated with the obtained medium with/without the presence of a combination of neutralizing antibodies against G-CSF (50ng/ml), GM-CSF (600pg/ml), IL-6 (4ng/ml) and sICAM-1 (10ng/ml). The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05 (one-way ANOVA followed by Bonferroni).

The presence of a combination of four antibodies against G-CSF, GM-CSF, IL-6 and sICAM-1 completely prevented ACA19$^-$ cell adhesion induced by the conditioned medium from galectin-3-treated HMVEC-Ls (Fig. 4.12).

### 4.4.9 Investigation of the effect of the presence of a combination of recombinant cytokines on cancer cell-endothelial adhesion

To further determine whether the increased secretion of cytokines by HMVEC-Ls in response to galectin-3 was responsible for the galectin-3 mediated cancer cell-endothelial adhesion, recombinant cytokines were included in the adhesion assessment. The presence of a combination of G-CSF, GM-CSF, IL-6 and sICAM-1 in a concentration similar to that in the conditioned-medium from 1µg/ml galectin-3-treated HMVEC-Ls was treated with HMVEC-Ls monolayers for 1 hr before 1 hr cancer cell-endothelial adhesion.

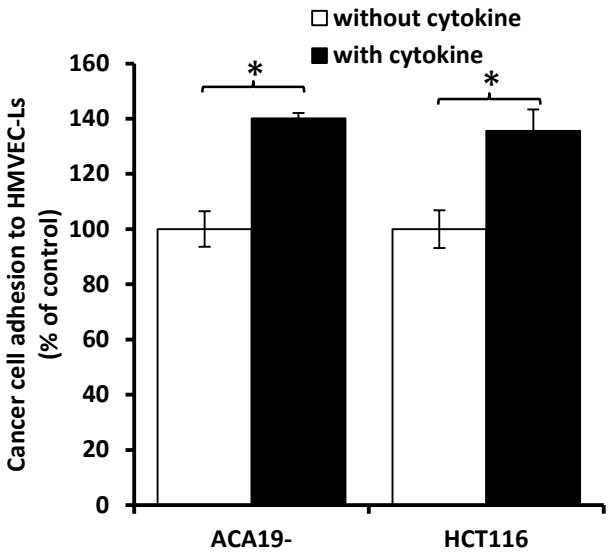


**Fig. 4.13: Recombinant cytokines increase cancer cell-endothelial adhesion.** ACA19$^-$ and HCT116 cell adhesion to HMVEC monolayer was assessed in the presence or absence of a combination of G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml). The data are expressed as mean ± SD of triplicate determinations of three independent experiments. p*<0.05 (unpaired t test).

It was found that the combination of cytokines induced a similar increase of ACA19$^-$ (40.1±6.8%) and HCT116 (35.6±7.7%) cell adhesion (Fig. 4.13) to that of the conditioned medium from galectin-3-treated HMVEC-Ls. These results indicate that galectin-3-induced secretion of G-CSF, GM-CSF, IL-6 and sICAM-1 by HMVEC-Ls is essential for galectin-3-induced MUC1-negative cancer cell adhesion to endothelial cells.

### 4.4.10 Investigation of the effect of galectin-3-induced cytokine secretion on metastasis in mice

The effect of circulating galectin-3 on cytokine secretion was further analyzed *in vivo* in nude mice. Five µg/mouse (2.5µg/ml, assuming a 2ml blood volume) galectin-3, which is a pathological galectin-3 concentration seen in cancer patients with metastasis (321), was injected intravenously into the tail vein. Animals were sacrificed at 0, 24 and 48 hr and the serum concentrations of G-CSF, GM-CSF, IL-6 and sICAM-1 were determined by individual cytokine ELISAs.

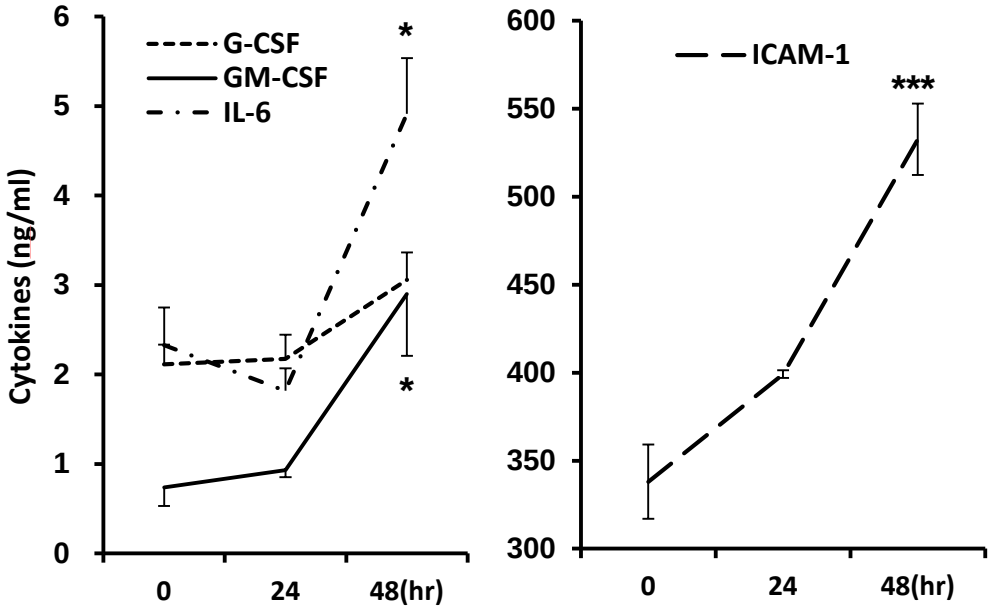


**Fig. 4.14: Galectin-3 induces cytokine secretion in mice.**

Galectin-3 induces cytokine secretion *in vivo*. Mice (three mice per group) were injected with 5µg/mouse recombinant galectin-3 via the tail vein. The animals were sacrificed at 0, 24 and 48 hr and serum concentrations of sICAM-1, G-CSF, GM-CSF and IL-6 cytokine were determined. P*<0.05, **p<0.01, ***p<0.001 (One-way ANOVA followed by Bonferroni).

There was a significant increase in G-CSF, GM-CSF, IL-6 and sICAM-1 levels. A 45.1±14.4% increase of G-CSF, 293.7±93.7% of GM-CSF, 111.2±26.7% of IL-6 and 57.6±6.0% of sICAM-1 in the sera was observed 48 hr after galectin-3 injection (Fig. 4.14 and Table 4.3). This provides strong evidence of an influence of circulating galectin-3 on secretion of these cytokines *in vivo*.

**Table 4.3 galectin-3 induce cytokine secretion in mouse *in vivo***

| | **Time after Injection (hr)** | **Increase compare to time 0 (%)** | **Standard Deviation** | **P value** |
|---|---|---|---|---|
| **G-CSF** | 24 | 2.83 | 12.8 | 1 |
| | 48 | 44.56 | 14.5 | 0.142 |
| **GM-CSF** | 24 | 26.21 | 10.8 | 1 |
| | 48 | 292.63 | 93.8 | 0.033 |
| **IL-6** | 24 | -21.94 | 10.8 | 10.021 |
| | 48 | 111.01 | 26.7 | 0.021 |
| **ICAM-1** | 24 | 19.09 | 0.6 | 0.216 |
| | 48 | 57.59 | 6.0 | 0.001 |

## 4.5 Discussion

The experiments described here show that the presence of galectin-3 in the pathological concentrations seen in cancer patients induce dose- and time-dependent secretion of metastasis-promoting cytokines IL-6, sICAM-1, G-CSF and GM-CSF from the vascular endothelium. The secretion of these cytokines autocrinely/paracrinely increases the adhesion of MUC1-negative cells to vascular endothelial cells *in vitro*.

Vascular endothelium is an extraordinarily complex network of cells with multitudes of distinct anatomic, metabolic and immunologic properties. Endothelial cells can produce many diverse biologically active molecules such as cytokines and growth factors. Since endothelium forms the internal lining of the entire vascular system, the production of such active molecules can have profound influence either directly or indirectly on the processes of tumourigenesis, angiogenesis, progression and metastasis, locally and symmetrically.

Cytokines are multi-functional proteins. Pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 are now well recognized as important regulators in cancer progression, metastasis and tumor immune suppression. These cytokines can regulate tumor cell behaviors directly or indirectly through regulation of the tumor microenvironment in an autocrine or paracrine manner. This study shows that circulating galectin-3 induces secretion of four cytokines from the blood vascular endothelium to the circulation including pro-inflammatory cytokine IL-6 and G-CSF.

IL-6 is a pleiotropic and pro-inflammatory cytokine and is known to be secreted by various cell types including fibroblasts, macrophages, and lymphocytes, epithelial and endothelial cells. IL-6 plays diverse roles as a regulator of the acute inflammatory response and as a growth and survival factor. IL-6 binds its receptor IL-6Rα on the cell surface that recruits the cell membrane gp130, leading to the formation of a signaling complex. This results in activation of at least three signaling pathways of JAK/STAT, Ras/ERK and PI-3/Akt (391), leading to the unregulated expression of a large variety of genes involved in cell proliferation and growth.

In cancer, high serum levels of IL-6 correlate with cancer severity, presence of metastasis and poor prognosis of many types of cancers including colorectal (392), prostate (393), breast(394) and gastric cancer(395). IL-6 can stimulate the release of angiogenesis-promoting factors such as VEGF and bFGF and promote angiogenesis (396). IL-6 expression and secretion can increase epithelial-mesenchymal transition in cancer by promoting E-cadherin expression or JAK/STAT-3 signaling (397). As a pro-inflammatory cytokine, IL-6 can activate the Stat-3 signaling in regulator T cells and help tumor cells escaping from immune surveillance (59). Recently it has been revealed that IL-6 production in a primary tumor can promote the recruitment of circulating tumor cells back to their primary tumor, creating a process called tumor self-seeding that accelerates tumor growth, angiogenesis and stromal cell recruitment(398). The IL-6-mediated activation of Stat-3 signaling in inflammatory cells can also lead to transcriptional activation of NF-Kappa B for secreting additional IL-6 and IL-8 to act on tumor cells, thus generating a positive feedback loop between immune cells and tumor cells that further stimulates the tumor cell progression and

metastasis (399). As a result of such divergent influences of IL-6 on tumor progression and metastasis promotion, inhibition of IL-6-mediated cell signaling has been the subject of intense investigation in the past few years in cancer treatment. Several phase I and II clinical trials using either anti-IL-6 antibody or IL-6 inhibitors, alone or in combination with other therapies, are currently under way (391, 400, 401).

G-CSF is a cytokine that stimulates bone marrow to produce granulocytes. It is widely used for the treatment of congenital neutropenia and to aid haematopoietic recovery following thermotherapy for a range of solid tumors. G-CSF binds to its cell surface receptor G-CSFR and induces receptor multimerization and activation of several intracellular signaling pathways of JAK/STAT, Ras/ERK and PI3K/Akt (402). Serum G-CSF levels have been reported to be higher in urothelial cancer patients than in healthy people and patients with an undetectable level of serum G-CSF have higher disease-specific five-year survival rates than those with an elevated level of serum G-CSF(403). Recently G-CSF has been shown to be able to mobilize Ly6G+Ly6C+ granulocytes in pre-metastatic tissues in distant organs before the arrival of tumor cells, facilitate subsequent tumor cell homing at this newly-created pre-metastatic environment and promote tumor cell migration, angiogenesis and metastasis (404). Direct injection of recombinant G-CSF into the tail vein of nude mice for five consecutive days before and after tumor cell injection has been shown to cause significant increase of the lung metastasis of animals injected not only with metastatic but also with non-metastatic breast cancer cells (404).

GM-CSF is a cytokine that, like G-CSF, stimulates the bone marrow to produce granulocytes. It is often used to stimulate the production of white blood cells following chemotherapy in cancer patients. Serum GM-CSF levels have been shown to be higher in breast cancer patients than in healthy people (405). The presence of GM-CSF promotes the invasiveness of lung cancer cells and prolongs tumor cell survival by activation of MEK/ERK and PI3K/Akt signaling *in vitro* (406)and in lung cancer xenograft (407).

sICAM-1 is a soluble form of the transmembrane cell adhesion molecule ICAM-1. ICAM-1 is weakly expressed in leukocytes, epithelial and resting endothelial cells and is up-regulated upon stimulation by inflammatory mediators such as cytokines and LPS. ICAM-1 binds to Mac-1 (macrophage adhesion ligand-1) and integrin LFA-1 (leukocyte function-associated antigen-1) and promotes cell-cell interactions. Higher sICAM-1 levels are seen in various cancers (408) including breast, gastrointestinal, lung, stomach, melanoma, ovary and bladder cancer patients (409) and in particular those with metastasis(410). High serum levels of sICAM-1 correlate significantly with TNM stage and lymph node in colorectal cancer patients (411) and a high pre-operative level of sICAM-1 has been shown to be an independent prognostic marker for stage II colorectal cancer (412). The circulation of sICAM-1 inhibits T cell interaction with tumour cells (413), blocks NK cell-mediated toxicity of tumour cells (414) and promotes tumour cell escape from immune surveillance.

Thus, IL-6, G-CSF, GM-CSF and sICAM-1 can all have a divergent and positive influence on cancer progression and metastasis. The increased secretion of these

cytokines in the blood circulation by the blood vascular endothelium in response to the increased circulation of galectin-3 in cancer patients will therefore have profound influence on cancer progression and metastasis, not only as regards the cancer cell adhesion and angiogenesis shown in this study but as regards other behaviors of tumor cells as well as the tumor micro-environment locally, remotely and systematically.

Although IL-6, G-CSF, GM-CSF and sICAM-1levels have all been shown previously to be increased in cancer patients, the source of these increased circulating cytokines is unclear. Mice bearing human colon cancer MC-38 cells did not show a significant increase of circulating IL-6, indicating that tumor-derived IL-6 may not have a measurable effect on systemic IL-6 levels (415). Mice bearing human MDA-MB-231 tumors on the other hand showed increased plasma levels of both human and mouse G-CSF, suggesting that host cells are a significant source of circulating G-CSF(404). The discovery that circulating galectin-3 increases the secretion of IL-6, G-CSF, GM-CSF and sICAM-1 in the blood vascular endothelium supports an important contribution of the host cells such as the blood vascular endothelium to the increased circulation of these cytokines in the circulation of cancer patients.

# CHAPTER 5 Investigation of the influence of galectin-3-induced endothelial secretion of cytokines on endothelial behaviors relevant to angiogenesis and metastasis

## 5.1 Hypothesis and Aim

To explore the hypothesis that galectin-3-induced cytokine secretion affects endothelial cell behavior in a way likely to promote metastasis.

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## 5.2 Introduction

Cytokines such as IL-6 and G-CSF are multifunctional proteins. Their pleiotropic actions not only include effects on the immune system and modulation of inflammatory responses but also include effects on progression of various steps of tumor growth, angiogenesis and metastasis (94). The discovery of the increased secretion of IL-6, G-CSF, GM-CSF and sICAM1 by vascular endothelium in its response to galectin-3 implies that the galectin-3-induced secretion of these cytokines may promote these pro-angiogenic and pro-metastasis steps in cancer.

## 5.3 Methods

### 5.3.1 Assessments of cell surface adhesion molecules by flow cytometry

Sub-confluent HMVEC-Ls were treated with galectin-3 (1.5μg/ml) for 24 hr at 37℃. The cells were released from the culture flasks by trypsinization. After the cells had been washed once with PBS, 5ml 2% paraformaldehyde was added in to fix for at least 25 minutes at room temperature. Then the cells were washed twice with 10ml PBS and centrifuged to remove the supernatants. After the addition of 0.5ml PBS, cells were counted and resuspended into $10^6$/ml with 5% goat serum/PBS for 30 minutes at room temperature on the roller platform. After removal of the supernatant following centrifugation, the cells were resuspended in 1% goat serum in PBS and 1ml/tube of the cell suspension was aliquoted into 1.5ml tubes. Antibodies against CD44 (1mg/ml), integrin $\alpha_5\beta_1$ (1mg/ml), E-selectin (1mg/ml), sICAM-1 (1mg/ml) and VCAM (1mg/ml) in PBS (1:400 dilution) were applied to the cells for 1 hr at room temperature on the roller (or overnight at 4℃). After two washes with PBS, FITC-conjugated secondary antibodies (1:400 in 1% BSA in PBS) were applied for 1 hr at room temperature. After three washes with PBS, the cells were resuspended in PBS in 0.5ml/tubes. The cell surface expression of CD44, integrin$\alpha_5\beta_1$, E-selectin, sICAM-1 and VCAM was analyzed by flow cytometry. Application of FITC-conjugated secondary antibody without the primary antibody was used as a negative control in all the flow cytometry analyses.

### 5.3.2 Assessment of endothelial tubule formation using an *in vitro* Angiogenesis Assay Kit

HMVEC-Ls (1 x $10^5$/well) were cultured in a 12-well plate at 37℃ for 24 hr before the introduction of 1.5μg/ml recombinant galectin-3 or BSA with or without the addition of a combination of anti-cytokine antibodies mixture (final concentration: G-CSF (25ng/ml), GM-CSF (300pg/ml), IL-6(2ng/ml) and sICAM-1 (5ng/ml) or recombinant cytokines (a combination of recombinant cytokines G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml)) for 24 hr. The conditioned medium was collected for the following assessment steps. The culturing time was precisely calculated so that the conditioned medium was fresh.

Cultrex RGF BME was thawed at 2-4℃ overnight in a refrigerator. The next day, 50μl of BME solution was aliquoted into the wells (three wells per group) of a 96-well plate on ice in the hood. The plate was centrifuged at 250 (**g*) for five minutes at 4℃ to make sure no air bubbles were trapped in the BME and then the plate was incubated at 37℃ for 45 minutes to make the gel solid.

Then 5μl of 2mM Calcein AM Working Solution was added to 5ml EBM-2 to make 2μM Calcein AM solution. Sub confluent HUVEC cells cultured in a T25 flask were incubated with the Calcein AM solution (5 ml per T25 flask) for 30 minutes at 37℃ in a $CO_2$ incubator before they were released by NECDS (2ml/each). The supernatant was discarded after centrifugation at 1000 (**g*) for 5 min. Then the cell pellet was resuspended with 200μl PBS and further diluted to 1 x $10^5$ cells /ml using the conditioned medium collected above, respectively for each group. Then 100μl of

diluted cells (1 x $10^4$ cells / well) were added slowly to each well of the 96 well-plate containing gelled BME above. The plate was then incubated at 37℃ in a $CO_2$ incubator for 24 hours before tube formation was analyzed as described in the general method chapter.

## 5.4 Results

### 5.4.1 Investigation of the effect of galectin-3 treatment on expression of cell surface adhesion molecules by HMVEC-Ls

To gain an insight into the mechanism of the galectin-3-induced, cytokine-mediated cell adhesion, we analyzed the expression of several common cell surface adhesion molecules on HMVEC-Ls after treatment of the cells with galectin-3. HMVEC-Ls cells were treated with galectin-3 (1.5μg/ml) for 24 hr before the expression of endothelial cell surface adhesion molecules (Integrin$\alpha_5\beta_1$, Integrin$\alpha_5\beta_3$, CD44, E-selectin, ICAM and VCAM) were analyzed by flow cytometry.

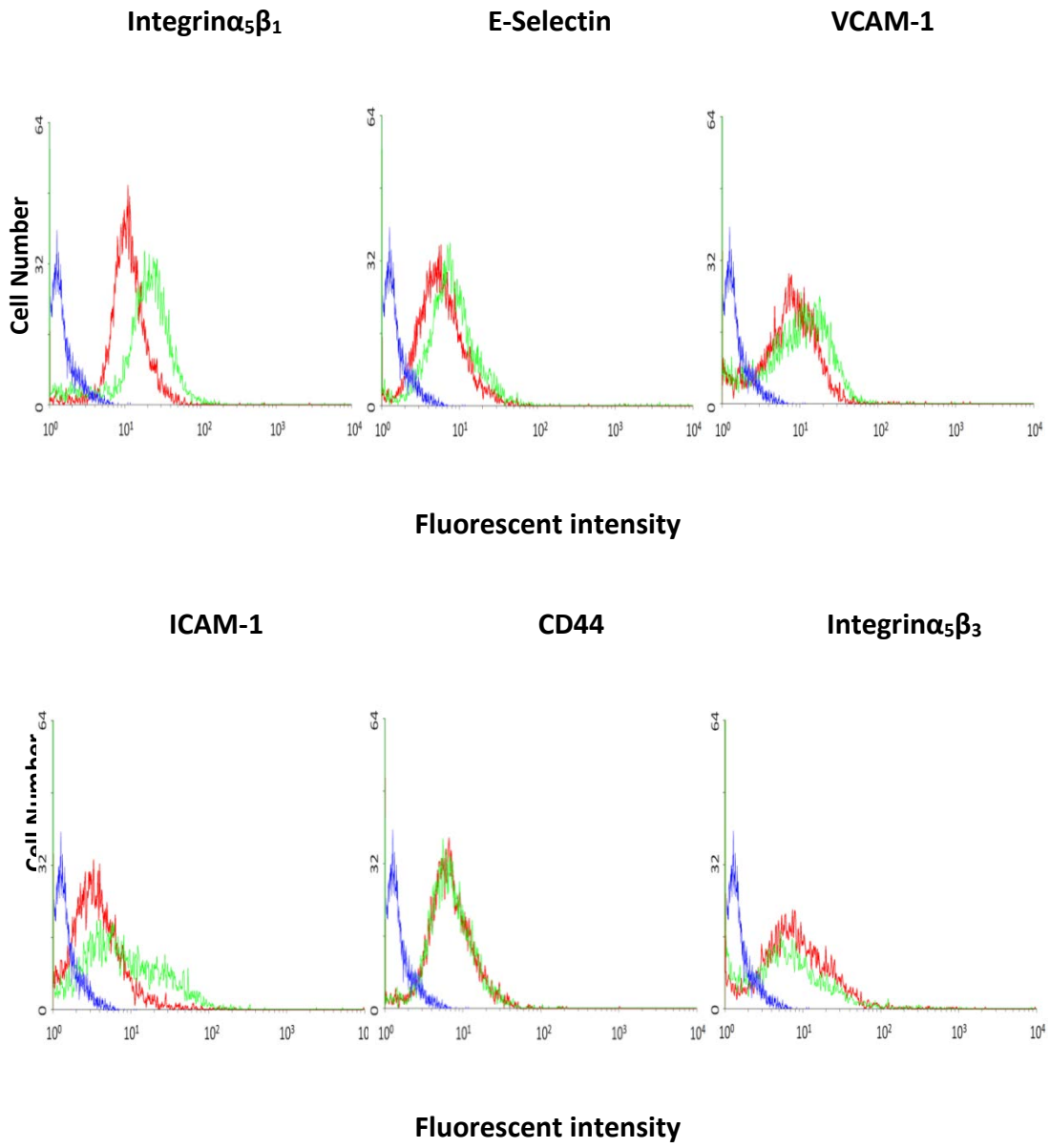



**Fig. 5.1: Galectin-3 induces expression of cell surface Integrin$\alpha_5\beta_1$, E-selectin, ICAM and VCAM but not Integrin$\alpha_5\beta_3$ or CD44.** The HMVEC-Ls monolayer was treated without (red) or with 1.5µg/ml galectin-3 (green) for 24 hr before the expression of HMVEC-Ls cell surface adhesion molecules was analyzed by flow cytometry (n=3). The IgG control is shown in blue.

It was found that HMVEC-Ls treated with galectin-3 (1.5ug/ml) for 24 hr showed increased expression of cell surface Integrin$\alpha_5\beta_1$ (43.1% (95%CI 40.9, 45.3)), E-

selectin (18.6% (95%CI 15.5, 21.7)), VCAM-1 (16.7% (95%CI 13.4, 20.0)) and ICAM-1 (32.7% (95%CI 29.4, 36.0)) whereas the expression of cell surface CD44 and integrin$\alpha_5\beta_3$ was not affected (n=3) (Fig. 5.1).

To determine whether the increased expression of these endothelial cell surface adhesion molecules in response to galectin-3 was linked with galectin-3-induced cytokine secretion, the expression of integrin$\alpha_5\beta_1$, the adhesion molecule that showed the most increase in response to galectin-3, was further analyzed. HMVEC-Ls cells were treated with galectin-3 (1.5µg/ml) in the presence or absence of 10µM lactose for 24 hr before the conditioned medium was collected. Fresh HMVEC-Ls cells were treated with the collected galectin-3-treated conditioned medium in the presence or absence of a combination of neutralizing antibodies against G-CSF (25ng/ml), GM-CSF (300pg/ml), IL-6 (2ng/ml) and sICAM-1 (5ng/ml) for 1 hr. Then the expression of endothelial cell surface adhesion molecule Integrin$\alpha_5\beta_1$ was analyzed by flow cytometry.

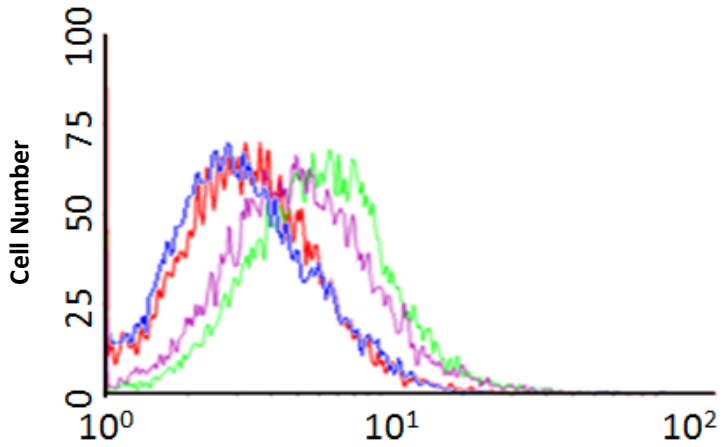


**Fig. 5.2: Galectin-3 induced expression of cell surface Integrin$\alpha_5\beta_1$ can be inhibited by the presence of lactose or a combination of neutralizing antibodies.** HMVEC-Ls monolayer was treated 1 hr with conditioned medium obtained from HMVEC-Ls treated with galectin-3 with the presence (blue) or absence of lactose (green) for 24 hr, or the HMVEC-Ls monolayer was treated 1 hr with conditioned medium obtained from HMVEC-Ls treated with galectin-3 for 24h and then a combination of neutralizing antibodies against G-CSF, GM-CSF, IL-6 and sICAM-1 (purple) was added in, before the expressions of HMVEC-Ls cell surface adhesion molecule Integrin$\alpha_5\beta_1$ were analyzed by flow cytometry (n=3). IgG control shows in red.

It is observed that a combination of neutralizing antibodies against G-CSF, GM-CSF, IL-6 and sICAM-1 in the culture medium resulted in 30.4% reduction of the galectin-3-mediated increase of integrin$\alpha_5\beta_1$ expression while pretreatment of lactose fully abolished the increased integrin$\alpha_5\beta_1$ expression that galectin-3 induced (Fig. 5.2).

To further determine whether the galectin-3 induced increased expressions of endothelial cell surface adhesion molecule integrin$\alpha_5\beta_1$ was mediated by galectin-3-induced cytokine secretion, HMVEC-Ls cells were incubated with a combination of recombinant G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml) at concentrations similar as that in the conditioned medium of galectin-3-treated HMVEC-Ls. Then the expression of endothelial cell surface adhesion molecule Integrin$\alpha_5\beta_1$ was analyzed by flow cytometry.

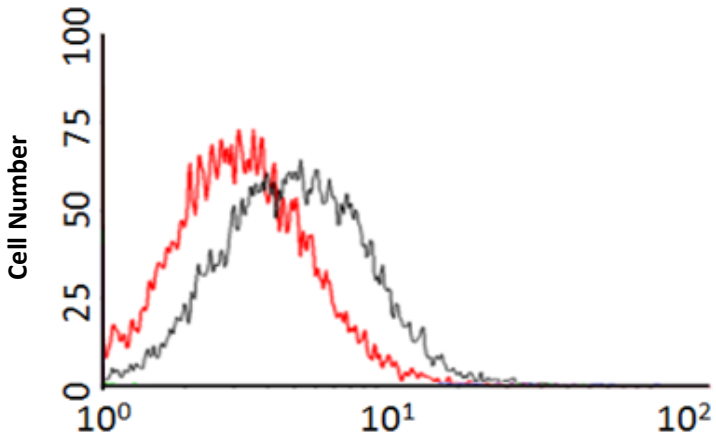



**Fig. 5.3: A combination of recombinant cytokines in concentration similar to that in the conditioned medium of galectin-3-treated HMVEC-Ls induces expression of cell surface Integrin$\alpha_5\beta_1$. The** HMVEC-Ls monolayer was treated without (red) or with a combination of recombinant G-CSF, GM-CSF, IL-6 and sICAM-1 at concentrations similar to that in the conditioned medium of galectin-3-treated HMVEC-Ls for 1 hr before the expression of HMVEC-Ls cell surface adhesion molecule Integrinα5β1 was analyzed by flow cytometry (n=3).

Furthermore, as was seen with recombinant galectin-3, incubation of HMVECs with a combination of recombinant G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml) concentrations similar to those in the conditioned medium of galectin-3-treated HMVECs caused a 40.3% increase in cell surface Integrin$\alpha_5\beta_1$ expression (Fig. 5.3). This indicates that the increased expression of endothelial cell surface adhesion molecules induced by galectin-3 is associated with the autocrine/paracrine actions of galectin-3-induced secretion of cytokines in endothelial cells.

To investigate the relative impacts of the four cytokines induced by galectin-3 on promoting the expression of endothelial cell surface adhesion molecules, the effects of these four cytokines on Integrin$\alpha_5\beta_1$ cell surface expression were assessed individually. HMVEC-Ls cells were incubated individually with recombinant G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) or sICAM-1 (500pg/ml) at concentrations similar to that in the conditioned medium of galectin-3-treated HMVEC-Ls. Then the expression of endothelial cell surface adhesion molecule Integrin$\alpha_5\beta_1$ was analyzed by flow cytometry.

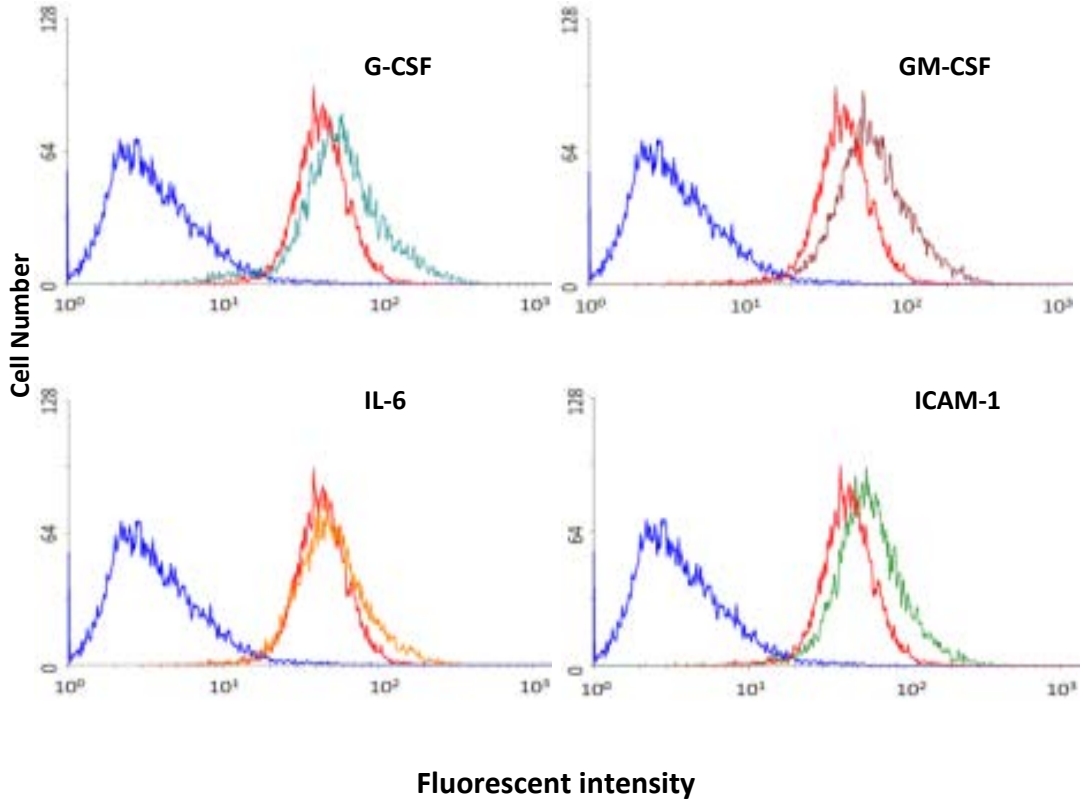



**Fig. 5.4: Each recombinant cytokine at concentrations similar to that in the conditioned medium of galectin-3-treated HMVEC-Ls induces expression of cell surface Integrin$\alpha_5\beta_1$. The** HMVEC-Ls monolayer was treated without (red) or with recombinant G-CSF (cyan), GM-CSF (brown), IL-6 (orange) and sICAM-1 (green) at concentrations similar to that in the conditioned

medium of galectin-3-treated HMVEC-Ls for 1 hr before the expression of HMVEC-Ls cell surface adhesion molecule Integrin$\alpha_5\beta_1$ was analyzed by flow cytometry (n=3). IgG control is shown in blue.

Each of these four cytokines increased Integrin$\alpha_5\beta_1$ expression on HMVEC-Ls, albeit to different extents. A 33.9% increase was observed for 2.5ng/ml G-CSF, 31.6% for GM-CSF (30pg/ml), 17.3% for IL-6 (200pg/ml) and 35.8% for ICAM-1 (500pg/ml) (Fig. 5.4). This suggests that the galectin-3-induced cytokines probably all make a contribution to the galectin-3-mediated increase in expression of endothelial cell surface adhesion molecules.

To investigate the relationship between increased expression of the endothelial cell surface adhesion molecules induced by galectin-3 and galectin-3-mediated adhesion of MUC1-negative cancer cells, we assessed the effect of antibodies against the cell adhesion molecules on galectin-3-mediated ACA19$^-$ adhesion.

Confluent HMVEC-Ls cell monolayers in 96 well plates were treated with galectin-3 (1.5µg/ml) for 24 hr and BSA was used as negative control. After 24 hr incubation, the conditioned medium in wells was collected. A combination of neutralization antibodies against Integrin$\alpha_5\beta_1$ (10µg/ml), E-selectin (10µg/ml), VACM-1 (10µg/ml) and ICAM-1 (10µg/ml) were added to the collected conditioned medium before fresh HMVEC-Ls were treated with them for 1 hr. Then, ACA19$^-$ at a

concentration of 5 x $10^4$/ml cell was used for adhesion to the treated HMVEC-Ls for 1 hr before assessment.

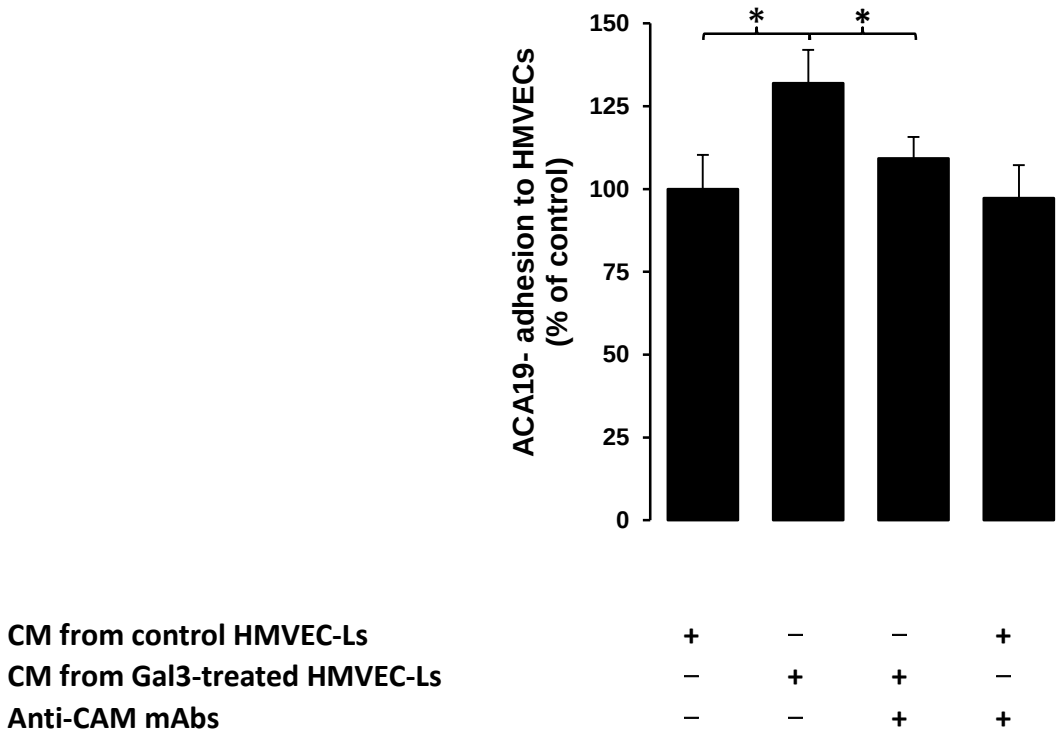



**Fig. 5.5: The presence of antibodies against the cell surface adhesion molecules inhibits galectin-3-mediated cancer cell adhesion.** The HMVEC-Ls monolayer was first treated with or without galectin-3 for 24 hr and then ACA19$^-$ cell adhesion to the HMVEC-Ls monolayer was assessed in the presence or absence of a combination of neutralization antibodies against Integrin$\alpha_5\beta_1$, E-selectin, VACM-1 and ICAM-1(n=3). P*<0.05 (One-way ANOVA followed by Bonferroni).

A combination of neutralizing antibodies against integrin$\alpha_5\beta_1$, E-selectin, VCAM-1, and ICAM-1 (10μg/ml for each) resulted in a 71.1% decrease of galectin-3-associated ACA19- cell adhesion to HMVECs (Fig. 5.5). Collectively, these results

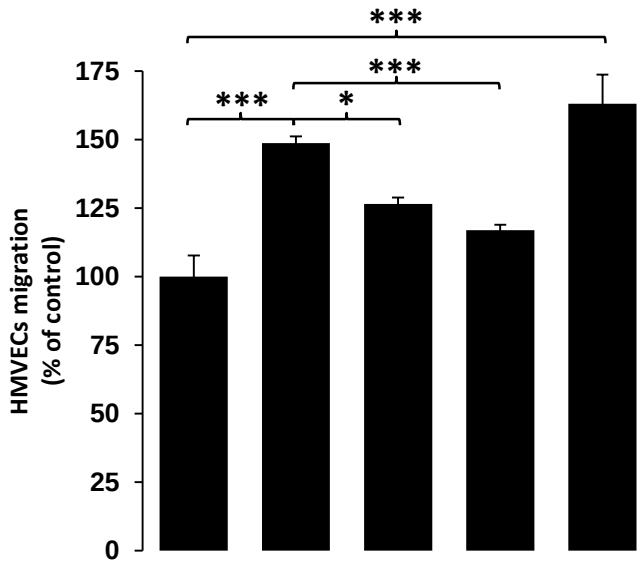
indicate that the cytokine-induced expression of the endothelial cell surface adhesion molecules in response to galectin-3 is responsible for the increased adhesion of MUC1-negative cells induced by galectin-3.

### 5.4.2 Investigation of effect of galectin-3 treatment on HMVEC-Ls cell migration

As pro-inflammatory cytokines such as IL-6 have been shown previously also to promote angiogenesis (391) and the galectin-3-induced secretion of cytokines from the vascular endothelium included the pro-inflammatory cytokines IL-6 and G-CSF, we further speculated that the release of these cytokines by the blood vascular endothelium in response to circulating galectin-3 might also influence endothelial behavior during angiogenesis. We therefore assessed the effect of galectin-3-induced cytokine secretion by HMVECs on endothelial cell migration through basement matrix proteins as well as on endothelial micro-tubule formation, two important components of the angiogenesis process.

Confluent HMVEC-Ls were treated with either BSA, or galectin-3 (1.5µg/ml) in the presence or absence of 10µM lactose for 48 hr. A fresh flask of HMVEC-Ls was harvested, washed with PBS, centrifuged and resuspended at the concentration of 1 x $10^7$cells/ml in EBM-2. The suspension was aliquoted (200µl/well) to sterile centrifuge tubes and diluted to 4 x $10^5$cells/ml with conditioned medium collected from the incubation with and without the addition of neutralizing anti-cytokine antibodies (a combination of neutralizing antibodies against G-CSF (25ng/ml), GM-

CSF (300pg/ml), IL-6 (2ng/ml) and sICAM-1 (5ng/ml)) or recombinant cytokines (a combination of recombinant cytokines G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml)). Then their invasion abilities through the basement matrix were assessed by the use of pre-set devices provided by the *In Vitro* Angiogenesis Assay Endothelial Cell Invasion Kit.




| | | | | | |
|---|---|---|---|---|---|
| **CM from BSA-treated HMVEC-Ls** | + | – | – | – | + |
| **CM from Gal-3-treated HMVEC-Ls** | – | + | + | + | – |
| **CM from Gal-3/Lac-treated HMVEC-Ls** | – | – | + | – | – |
| **Cytokine neutralizing antibodies** | – | – | – | + | – |
| **Recombinant cytokines** | – | – | – | – | + |

**Fig. 5.6: Galectin-3-induced cytokine secretion promotes endothelial cell migration.**

The HMVEC-Ls monolayer was treated with 1.5μg/ml BSA or galectin-3 in the presence or absence of lactose for 24 hr. The CM were collected and used for subsequent assessment of fresh HMVEC cell migration through basement matrix proteins in the presence or absence of a combination of neutralizing antibodies, or a combination of recombinant cytokines. The data are expressed as percentages compare to BSA-treated as control (n=3). P*<0.05, p***<0.001 (One-way ANOVA followed by Bonferroni).

The conditioned medium from 24-hr galectin-3 (1.5μg/ml)-treated HMVECs caused 48.8%±2.5% increase in migration of fresh HMVECs through basement matrix proteins compared with the BSA-treated control medium (Fig. 5.6). A combination of neutralizing antibodies against G-CSF (25ng/ml), GM-CSF (300pg/ml), IL-6 (2ng/ml) and sICAM-1 (5ng/ml) markedly reduced (65.2%±2.0%) galectin-3-associated HMVEC-Ls cell migration, suggesting that the galectin-3-induced secretion of these cytokines is responsible for the observed increase in HMVEC-Ls migration. This was further supported by a similar increase in HMVEC cell migration (63.1%±10.6%) when a combination of recombinant G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml) at similar concentrations to those of the conditioned medium from galectin-treated HMVECs was added to the culture.

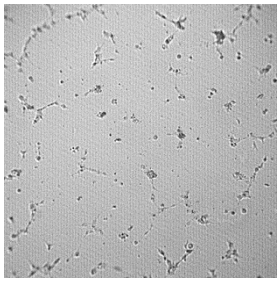
### 5.4.3 Investigation of effect of galectin-3 treatment on HUVEC cell tube formation

To test the impact of galectin-3 treatment on HUVEC cell tube formation, the conditioned medium from 24 hr galectin-3 (1.5μg/ml)-treated HMVEC-Ls was collected and used as culture medium with or without the addition of neutralizing anti-cytokine antibodies (a combination of neutralizing antibodies against G-CSF (25ng/ml), GM-CSF (300pg/ml), IL-6 (2ng/ml) and sICAM-1 (5ng/ml)) or recombinant cytokines (a combination of recombinant cytokines G-CSF (2.5ng/ml), GM-CSF

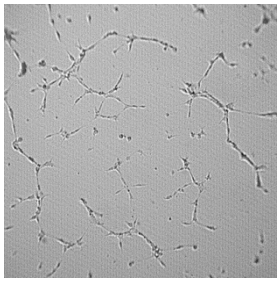
(30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml)) to culture HUVECs cells on the surface of matrix before the tubule formation ability of HUVEC cells was analyzed.

**A**

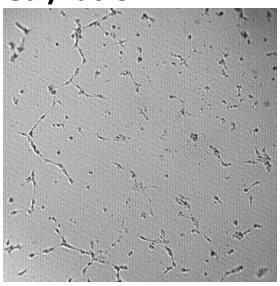
**BSA CM**



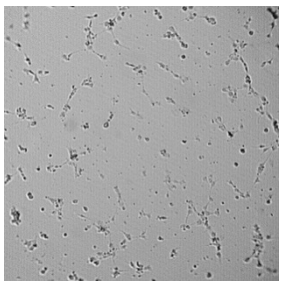
**Gal3 CM**



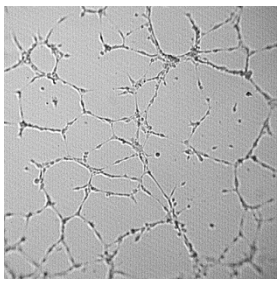
**Gal/Lac CM**



**Gal3 CM + Cytokine mAbs**



**BSA CM + rCytokines**



**B**

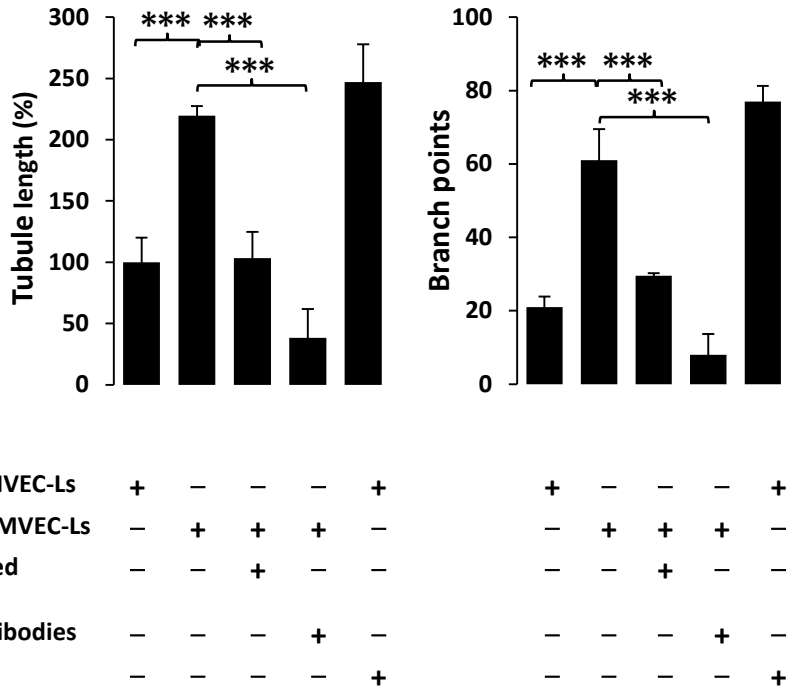



**Fig. 5.7: Galectin-3-induced cytokine secretion promotes endothelial tubule formation.**

HUVEC cells were cultured on top of matrix proteins in the conditioned medium (CM) obtained from HMVECs treated with BSA or galectin-3 (1.5µg/ml) for 24 hr in the presence or absence of 10µM lactose, with or without introduction to the conditioned medium of a combination of neutralizing antibodies against G-CSF, GM-CSF, IL-6 and sICAM-1 or a combination of recombinant G-CSF, GM-CSF, IL-6 and sICAM-1 for 24 hr at $37^0$C. Picture of each well was taken and representative pictures were shown (A), while tubule length and branch points (B) were quantified. The data are expressed as percentages compare to BSA-treated controls from three independent experiments, each in triplicate (n=3). P***<0.001 (One-way ANOVA followed by Bonferroni).

When the tubule formation ability of endothelial cells was analyzed, the HUVECs (the most commonly used endothelial cells for investigating *in vitro* tubule formation) cultured in the conditioned medium obtained from 24 hr galectin-3 (1.5µg/ml)-treated HMVECs showed significant increase in tubule length (219.7±7.7%) and branch points (329.9±52.6%) compared with HUVEC cells cultured in the conditioned medium from 24 hr BSA-treated HMVECs (Fig. 5.7). These effects were prevented when the galectin-3 inhibitor lactose was introduced as galectin-3 or a combination of neutralizing antibodies against G-CSF, GM-CSF, IL-6 and sICAM-1 was added to the conditioned medium. Moreover, introduction of a combination of recombinant G-CSF, GM-CSF, IL-6 and sICAM-1 to the BSA-treated control medium resulted in similar increases in HUVEC tubule length (247.1±30.7%) and formation of branch points (321.8±58.8%) to those induced by the galectin-3-conditioned medium. Together, these results suggest that galectin-3-induced secretion of cytokines from the vascular endothelium also promotes endothelial angiogenesis.

### 5.4.4 Investigation of effect of galectin-3 treatment on MMP secretion by HMVEC-Ls cells

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which are capable of degrading all kinds of extracellular matrix proteins, as well as carrying out a number of other bio-activities. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands and cytokine activation (416). MMPs are also largely responsible for endothelial cell invasion by digesting the

basement matrix (417). As several cytokines have been reported to induce endothelial secretion of MMPs, we investigated whether galectin3-induced secretion of cytokines increases endothelial cell secretion of MMPs using a transwell system. HMVEC-Ls were released from the culture flasks by trypsinization and suspended at 2 x $10^5$/ml with EBM-2 culture medium before application of 200µl/well to each well on the top chamber of a cell invasion device and 150µl/well serum-free EBM-2 was added to each well on the bottom chamber before incubation for 24 hr at 37℃. The medium in the top chamber wells was replaced with fresh EGM-2 medium after three hours to remove residual Trypsin. On the next day, the medium in the top chamber was replaced by medium containing 1.5µg/ml BSA (control) or recombinant galectins-3 respectively, and the serum-free EBM-2 at the bottom wells was also renewed. After 24 hr incubation at 37℃, the medium (1ml/well) in the bottom chamber wells was collected and the levels of MMP were analyzed with the RayBio Human MMP Array, which include MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, TIMP-1, TIMP-2, TIMP-4.

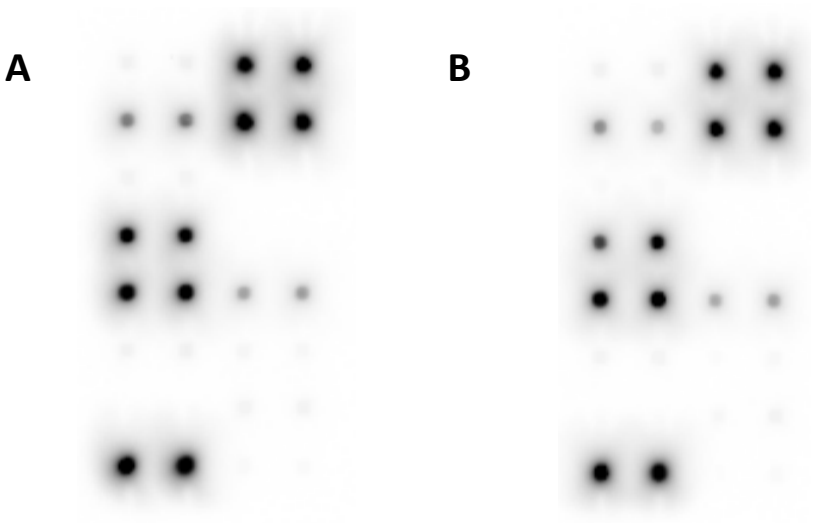


**Fig. 5.8: The presence of galectin-3 does not affect MMP secretion by HMVEC-Ls.**

The level of MMPs in the galectin-3-treated (A) or control wells of HMVEC-Ls in the chamber device were tested with the human MMPs array.

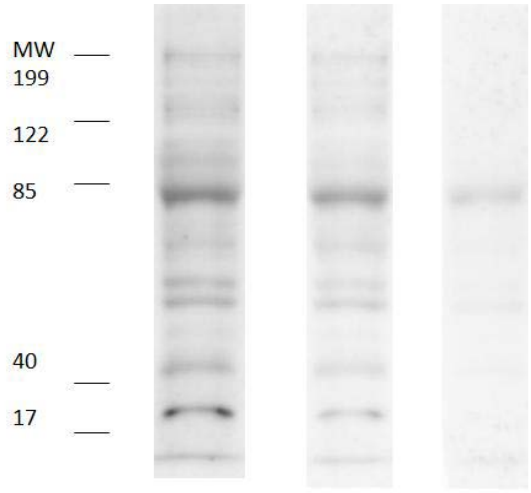
No detectable difference in the MMP levels in the medium from control or galectin-3-treated HMVEC-Ls was observed (n=2) (Fig. 5.8).

### 5.4.5 Investigation of galectin-3 binding to endothelial cells

To gain insight into the action of galectin-3-induced cytokine secretion by the endothelial cells, galectin-3 blotting of HMVEC-Ls was conducted.

Each flask of HMVEC-Ls cells was harvested by Trypsin (1ml Trypsin for each T25 flask) and lysed by 50µl 10% SDS sample buffer. Then 30µl of the cell lysis was loaded to each lane. In the blotting, the membranes were probed with or without 10µg/ml galectin-3 followed by probing with Biotinylated-anti galectin-3 as first antibody (10µg/ml) and Avidin-peroxidase conjugate bind to biotin of the biotinylated-anti galectin-3 (10µg/ml) as secondary antibody.

**Endothelial Cells (HMVEC-Ls):** **kDa**




| | | | |
|---|---|---|---|
| **Rhgalectin-3** | + | – | – |
| **Biotinylated-anti galectin-3** | + | + | – |
| **Avidin-peroxidase conjugate bind to biotin of the biotinylated-anti galectin-3** | + | + | + |

**Fig. 5.9: Galectin-3 recognizes several HMVEC-Ls proteins. A T25cm$^2$ flask of HMVEC-Ls cells was cultured to 80% confluence and released with Trypsin then lysed in sample buffer and the cell extracts were electrophoresed on an SDS PAGE (10%) electrophoresis gel.**

Several HMVEC-Ls proteins have proved to be recognized by galectin-3 when assessed by galectin-3 blotting (Fig. 5.9). By comparing first two lanes, it is shown that galectin-3 could recognize several proteins on the HMVEC-Ls. One of these proteins may represent the galectin-3 ligand responsible for galectin-3-mediated cytokine secretion. Flow cytometry could be further used to identify the specific galectin-3 binding ligand.

### 5.4.6 Investigation of effect of galectin-3 treatment on expression of phosphorylation in HMVEC-Ls cells

HMVEC-Ls cells were further investigated by means of a Human Phospho-Kinase Array that covers the 46 phosphorylation sites.

Each flask of the HMVEC-Ls monolayer was cultured in EBM-2 medium with the presence or absence of 1.0μg/ml galectin-3 or 1.0μg/ml BSA for 24 hr at 37℃ before being washed by PBS three times (10ml/time). The supernatants were removed completely before 0.4ml of Lysis Buffer 6 (from the kit) was added, mixed and left on a rocking platform for 30 minutes on ice to lyse the cells. After being centrifuged at 1000 (**g*) for 5 minutes, the supernatant was transferred into a clean test tube and immediately used for the Phospho-array.

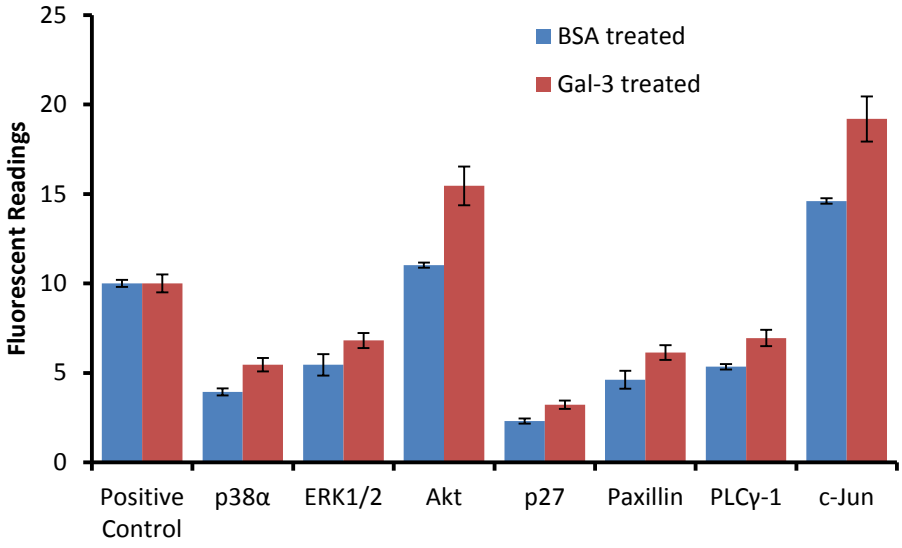



**Fig. 5.10: The presence of galectin-3 does not induce phosphorylation change in ACA19.**

Forty-six phosphorylation sites on the HMVEC-Ls cells treated with 1.0μg/ml BSA or with 1.0 μg/ml galectin-3 for 30 minutes were tested by the Human Phospho-Kinase Array. No statistical significance was observed.

No apparent phosphorylation difference was observed between HMVEC-Ls cultured with galectin-3 or BSA (Fig. 5.10).

### 5.4.7 Investigation of effect of galectin-3 induced cytokine secretion on metastasis formation in mice

To directly assess the influence of galectin-3-induced cytokine secretion on metastasis, we injected either PBS or a combination of GM-CSF, G-CSF, IL-6 and ICAM-1, at similar levels to those observed in mouse serum following galectin-3 injection. Eight mice were treated per experimental group. One mouse per group (PBS and cytokines injection) was sacrificed after four weeks and the rest were sacrificed six weeks post-injection and metastatic foci in the lungs were examined.

**A** **B**

**Control** **Treated**

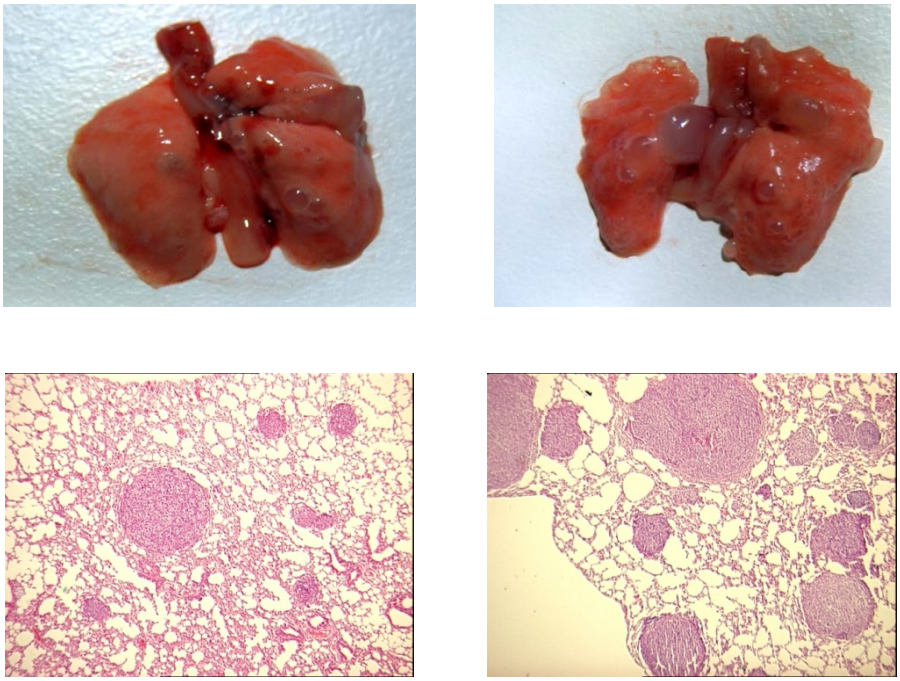


**Fig. 5.11: Injection of a combination of cytokines (G-CSF, GM-CSF, IL-6 and sICAM-1) followed by galectin-3 promotes metastasis (B) in mice compared with BSA-treated control group (A). The pictures represent 7 mice each group after six weeks' treatment.**

More microscopic metastatic nodules were observed in the cytokine-treated compared with the control mice after four weeks (15 vs. 0) and after six weeks (6 vs. 2) assessed by histology (Fig. 5.11). This result is not statistically significant ($p=0.151$). This may relate to the large standard deviation within the cytokine-treated group, which might be caused by failed injection.

### 5.4.8 Investigation of relationship between circulating galectin-3 and cytokine levels in colon cancer patients

To confirm whether the influence of galectin-3 on cytokine secretion observed in mice also held true for cancer patients, we investigated the relationship between serum galectin-3 and serum IL-6, G-CSF, GM-CSF and sICAM-1 concentrations in 50 colorectal cancer patients with or without clinically detectable metastasis (Table 5.1).

**Table 5.1: Concentrations and relationship between serum galectin-3 and IL-6, G-CSF, GM-CSF and sICAM-1 in colorectal cancer patients with the presence or absence of metastasis**

| | Serum concentrations Median (range, ng/ml) | Spearman's rho correlation coefficient | *p* |
|---|---|---|---|
| All patients (n=50) | | | |
| Gal-3 | 106.8 (7.5-5106.3) | | |
| IL-6 | 5.0 (2.1-287.8) | | |
| GM-CSF | 15.8 (5.4-833.3) | | |
| G-CSF | 43.0 (11.0-13266.0) | | |
| sICAM1 | 14442.0(7593.5-30235.9) | | |
| Patients without metastasis (n=39) | | | |
| Gal-3 | 82.0 (7.5-1603.9) | | |
| IL-6 | 5.2(2.7-72.1) | | |
| GM-CSF | 14.8(5.4-157.1) | | |
| G-CSF | 33.1 (11.1-3973.0) | | |
| sICAM1 | 15148.1(8414.1-30235.9) | | |
| Patients with metastasis (n=11) | | | |
| Gal-3 | 215.0 (30.9-5106.3) | | |
| IL-6 | 3.5(2.1-287.8) | | |
| GM-CSF | 15.8(12.0-833.3) | | |

| | | | |
|---|---|---|---|
| G-CSF | 29.0(15.0-13266.0) | | |
| sICAM1 | 12516.7(7593.5-21621.2) | | |
| All patients | | | |
| Gal-3 and IL-6 | | | |
| Gal-3 and GM-CSF | | 0.17 | 0.12 |
| Gal-3 and G-CSF | | 0.24 | 0.045 |
| Gal-3 and sICAM-1 | | -0.11 | 0.22 |
| Patients without metastasis | | | |
| Gal-3 and IL-6 | | 0.12 | 0.23 |
| Gal-3 and GM-CSF | | 0.11 | 0.26 |
| Gal-3 and G-CSF | | 0.19 | 0.13 |
| Gal-3 and sICAM-1 | | -0.056 | 0.37 |
| Patients with metastasis | | | |
| Gal-3 and IL-6 | | 0.51 | 0.05 |
| Gal-3 and GM-CSF | | 0.22 | 0.26 |
| Gal-3 and G-CSF | | 0.55 | 0.04 |
| Gal-3 and sICAM-1 | | 0.73 | 0.005 |

Serum concentrations of IL-6, G-CSF, GM-CSF and sICAM-1 in 50 colorectal cancer patients with and without clinically detectable liver metastasis were determined by galectin-3 ELISA. The relationship between serum galectin-3 and each of those cytokines was analyzed by Spearman correlation analysis.

A significant correlation between circulating galectin-3 concentration was observed with G-CSF levels ($p<0.05$) but not with the other three cytokines when all patients were considered together (Table 5.1). When, however, serum galectin-3 and cytokine levels were further analyzed in the patients who did not have metastasis and those with metastasis, significant correlations of galectin-3 levels were observed with G-CSF ($p=0.04$), IL-6 ($p=0.05$) and sICAM-1 ($p=0.005$) in patients with metastasis but not in those without metastasis (Table 5.1). This further supports an active role

of circulating galectin-3 in secretion of these metastasis-promoting cytokines in cancer patients.

## 5.5 Discussion

The experiments described here show that galectin-3-induced cytokines promote expression of cell surface adhesion molecules, as well as endothelial cell migration and micro-vascular tubule formation during angiogenesis. Thus, circulating galectin-3 induces the secretion from blood vascular endothelium of several cytokines including pro-inflammatory cytokines that promote cancer cell adhesion and angiogenesis. Such a conclusion is clearly supported by the observed correlation between circulating galectin-3 and these cytokines in colorectal cancer patients who suffer from metastasis. Galectin-3-mediated secretion of IL-6, G-CSF, GM-CSF and sICAM-1 from endothelium increases the expression of the endothelial cell surface adhesion molecules integrin$\alpha_5\beta_1$, E-selectin, VCAM-1 and ICAM-1.

ICAM-1 and VCAM-1 are members of the immunoglobulin superfamily and each carries a different number of extracellular immunoglobulin-like domains. Selectins are transmembrane glycoproteins that contain a lectin domain for binding to ligands, an epidermal growth factor-like domain and a series of short consensus repeats (SCR) in the extracellular region. Each selectin differs in the number of SCR domains. Integrins are transmembrane adhesion molecules and each is composed of one α and one β chains. There are at least 20 different α chains and nine different β chains. These cell surface adhesion molecules are responsible for recruiting leukocytes onto the vascular endothelium before extravasation to the injured tissues in inflammation. The expressions of those cell surface adhesion molecules are also

believed to be crucial in adhesion of disseminating tumor cells to the blood vascular endothelium in cancer cell haematogenous metastasis (409, 418).

Earlier studies have shown that pro-inflammatory cytokines such as TNFα and IL-1 can induce endothelial expression of cell surface adhesion molecules that increase the lodging of circulating tumor cells on the capillary bed both *in vitro* and *in vivo* (419). For example, intravenous injection of IL-1β into C57BL/6 mice increased the endothelial expressions of E-selectin, ICAM-1 and VCAM-1 in the hepatic microvasculature, causing a twofold reduction of B16F1 melanoma cell velocity in the blood circulation when they were subsequently injected (420). It has been postulated that induction of cell surface adhesion molecules by pro-inflammatory cytokines may partly account for the promotion of inflammation in cancer progression. Different inflammatory cytokines can influence the expression of different cell adhesion molecules in different cells. TNFα and LPS for example induce endothelial cell expression of E-selectin, ICAM-1 and VCAM-1 whereas Il-1β showed no effect on expression of these cell adhesion molecules (421).

The presence of exogenous galectin-3 in the culture medium, albeit at supra-pathological concentrations, has been previously shown to induce endothelial cell morphogenesis (422) and enhance VEGF- and bFGF-mediated angiogenesis by binding to the N-glycans of endothelial-associated integrin$\alpha_5\beta_3$ (423). As clustering of galectin-3 with its ligands can enhance the galectin-3 binding affinity as much as 10,000-fold (333), the effect of galectin-3 on VEGF- and bFGF-mediated angiogenesis demonstrated previously with higher than pathological concentrations of galectin-3

may also be functionally relevant in the blood circulation and contribute to metastasis promotion.

Several endothelial proteins seem to be recognized by galectin-3 when assessed by galectin-3 blotting. It is likely that one of these proteins represents the galectin-3 binding ligand responsible for galectin-3-mediated endothelial secretion of cytokines.

Thus, the increased circulation of galectin-3 in the bloodstream of cancer patients has several important and distinctive influences on cancer metastasis. It can interact directly with disseminating tumor cells through TF/MUC1 and increases cancer cell heterotypic adhesion (388) and homotypic aggregation (381). It can also interact with endothelial cells and induce endothelial secretion of metastasis-promoting cytokines and indirectly enhance metastasis. The increased circulation of galectin-3 in the bloodstream of cancer patients is therefore an important metastasis promoter. Targeting the actions of circulating galectin-3 represents a very promising therapeutic strategy to reduce metastasis and improve cancer survival.

# CHAPTER 6 Investigation of the effect of circulating galectin-2, -4 and -8 on cytokine secretion by blood vascular endothelium

## 6.1 Hypothesis and Aim

To assess the hypothesis that circulating galectin-2, -4 and -8, whose levels are also increased in cancer patients, may affect secretion of cytokines by vascular endothelium in a way likely to promote metastasis.

## 6.2 Introduction

Galectins are a family of 15 mammalian galactoside-binding proteins that share a consensus amino acid sequence in their carbohydrate recognition domains (CRDs)(171). Recent studies in our laboratory have shown that the levels of galectin-2, -4 and -8, like that of galectin-3, are all significantly increased in colon and breast cancer patients and particularly those with metastasis (321). Galectin-2 is a prototype galectin which contains only one CRD in its polypeptide sequences and galectin-4 and -8 are tandem-repeat type galectins which contain two CRDs in a single polypeptide chain. Although their structures are different from galectin-3, the only chimera-type galectin composed of a non-lectin domain linked to a CRD, they are all able to bind to galactoside-terminated glycans and form lattice structures on the cell surface. As circulating galectin-3 has been shown to induce secretion of cytokines by the blood vascular endothelium, we investigated whether those

galectin members whose levels are also increased in cancer patients also affect the endothelial secretion of cytokines.

## 6.3 Materials and Methods

### 6.3.1 Cytokine determination by Human Cytokine Array

Eighty per cent confluent HMVEC-Ls were released from the culture flasks by trypsinization and suspended at 1 x $10^5$/ml with EBM-2 medium. HMVEC-Ls suspensions were cultured in a six-well plate (2ml/well) at 37℃ for 24 hr before introduction of 1.5μg/ml recombinant galectin-2, -4, -8 or BSA for 24 hr. The conditioned media were collected and the concentrations of cytokines in the culture media were tested with Human Cytokine Protein Array as described in Chapter 3.

### 6.3.2 Cytokine determination by ELISA

HMVEC-Ls were released from the culture flasks by trypsinization and suspended with EBM-2 medium at 1 x $10^5$ cells/ml. HMVEC-Ls suspensions were added to a 12-well plate (1ml/well) and cultured for 24 hr before introduction of 1.5 μg/ml recombinant galectin-2, -4, -8 or BSA for 24 hr. The conditioned medium was collected and the concentrations of cytokines in the conditioned medium were analyzed by cytokine ELISA kits as in chapter 3.

### 6.3.3 Investigation of effect of galectin-2, -4 or -8 on cancer cell-endothelial adhesion-1

HMVEC-Ls were released from the culture flasks by trypsinization and suspended with EBM-2 medium (at 2 x $10^5$ cells/ml) before application of 200µl/well to each well of a 96-well plate. The medium in each well was replaced with 150 µl fresh EBM-2 medium after three hours. The cells were incubated until the formation of a cell monolayer (one day) before they were treated with 1.5µg/ml of galectin-2, -4, -8 or BSA in the presence or absence of lactose (10µM) for 24 hr. The HMVEC monolayer was then washed and used for subsequent assessment of cancer cell adhesion.

ACA19$^-$ and HCT116 cancer cells were detached from the culture plates with NECDS, washed and resuspended in serum-free DMEM medium (for ACA19 or McCoy's 5a medium for HCT116) at 5x$10^6$ cells/ml. The ACA19$^-$ and HCT116 cancer cells were labeled with Calcein AM Cell Labeling Solution (10 µl for 1 ml of cell suspension) for 30 minutes. The cells were washed and resuspended in serum-free medium (at 5 x $10^4$ cells/ml) before application of 100 µl to the HMVEC monolayer. After 1 hr of culture, the HMVEC monolayer was washed and the endothelial cell-associated fluorescence was measured.

### 6.3.4 Assessment of the effect of galectin-2, -4 or -8 on cancer cell-endothelial adhesion-2

To the HMVEC-Ls cells used for obtaining the conditioned medium, HMVEC-Ls were released from the culture flasks by trypsinization and suspended with EBM-2 medium at 2 x $10^5$/ml before application of 2 ml to each well of six-well plates. The medium in each well is replaced with 150 µl fresh EGM-2 medium after three hours. The cells were incubated for one day until the formation of a cell monolayer, when they were treated with 1.5µg/ml of galectin-2, -4, -8 or BSA in the presence or absence of lactose (3mM) for 24 hr. The culture medium (conditioned medium) was collected for use in the next step.

HMVEC-Ls were released from the culture flasks by trypsinization and suspended with EBM-2 medium at 2 x $10^5$/ml before application of 200µl to each well of a 96-well plate. The medium in each well was replaced with 150µl fresh EGM-2 medium after three hours. The cells were incubated until the formation of a cell monolayer for one day. Before the adhesion, the HMVEC monolayer was washed and incubated for 1 hr with conditioned medium obtained earlier. Then the HMVEC-Ls monolayers were washed and used for subsequent assessment of cancer cell adhesion.

ACA19$^-$ or HCT116 cancer cells were detached from the culture plates with NECDS, washed and resuspended in serum-free DMEM medium (McCoy's 5a medium for HCT116) at 5x$10^6$ cells/ml. The ACA19$^-$ and HCT116 cancer cells in 1 ml were labeled with 10µl Calcein AM Cell Labeling Solution for 1 hr, washed and

resuspended in serum-free medium at $5x10^4$/ml before application of $5x10^3$/well to the HMVEC monolayer for 1 hr at 37℃. The HMVEC monolayer was washed and the endothelial cell-associated fluorescence was measured.

### 6.3.5 Assessment of cell surface adhesion molecules by flow cytometry

Sub-confluent HMVEC-Ls were treated with galectin-2, -4 or -8 (1.5μg/ml) for 24 hr at 37℃. The cells were released from the culture flasks by trypsinization. After they had been washed once with PBS 5ml 2% paraformaldehyde was added to fix the cells. Then the cells were washed twice and centrifuged to remove the supernatants. After addition of 0.5ml PBS, cells were counted and resuspended in $10^6$/ml with 5% goat serum/PBS for 30 minutes at room temperature on the roller. After removal of the supernatant following centrifugation, the cells were resuspended in 1% goat serum in PBS and aliquoted to 1ml/tube in 1.5ml tubes. Antibodies against CD44 (1mg/ml), integrin$\alpha_5\beta_1$ (1mg/ml), E-selectin (1mg/ml) and VCAM (1mg/ml) in PBS (1:400 dilution) were applied to the cells for 1 hr at room temperature on the roller (or overnight at 4℃). After two washes with PBS, FITC-conjugated secondary antibodies (1:400 in 1% BSA in PBS) were applied to the cells for 1 hr at room temperature. After three washes with PBS, the cells were resuspended in PBS in 0.5ml/tubes. The cell surface expression of CD44, integrin$\alpha_5\beta_1$, E-selectin and VCAM were analyzed by flow cytometry. FITC-conjugated secondary antibody without the primary antibody was used as a negative control in all the flow cytometry analyses.

### 6.3.6 Assessment of endothelial tubule formation

HMVEC-Ls (1 x $10^5$/well) were cultured in a 12-well plate at 37℃ for 24 hr before introduction of 1.5μg/ml recombinant galectin-2, -4, -8 or BSA with or without the addition of a combination of anti-cytokine antibodies (final concentration: G-CSF(5ng/ml), IL-6(3ng/ml), GROα(20ng/ml) and MCP-1(20ng/ml)) for 24 hr. The conditioned medium was collected for the following assessment steps. The culturing time was precisely calculated so that the conditioned medium was fresh.

Sub confluent HUVEC cells cultured in T25 flasks were incubated with the Calcein AM solution (5 ml per T25 flask) for 30 minutes at 37℃ in a $CO_2$ incubator before they were released by NECDS (2ml/each), the supernatant was discarded after centrifugation at 1500 (x g) for 5 min. Then the cell pellet was resuspended by 200μl PBS and further diluted to 1 x $10^5$ cells /ml using each group of the conditioned medium collected above. 100μl of diluted cells (1 x $10^4$ cells / well) was added slowly onto each well of the 96 well plates containing gelled BME. The plate was then incubated at 37℃ in a $CO_2$ incubator for 24 hours before tube formation was analyzed as described in the chapter 3.

## 6.4 Results

### 6.4.1 Investigation of effect of galectin-2, -4 or -8 on cytokine secretion by HMVEC-Ls

The effect of galectin-2, -4 and -8 on cytokine secretion by endothelial cells was first assessed with HMVEC by Human Cytokine Protein Array.

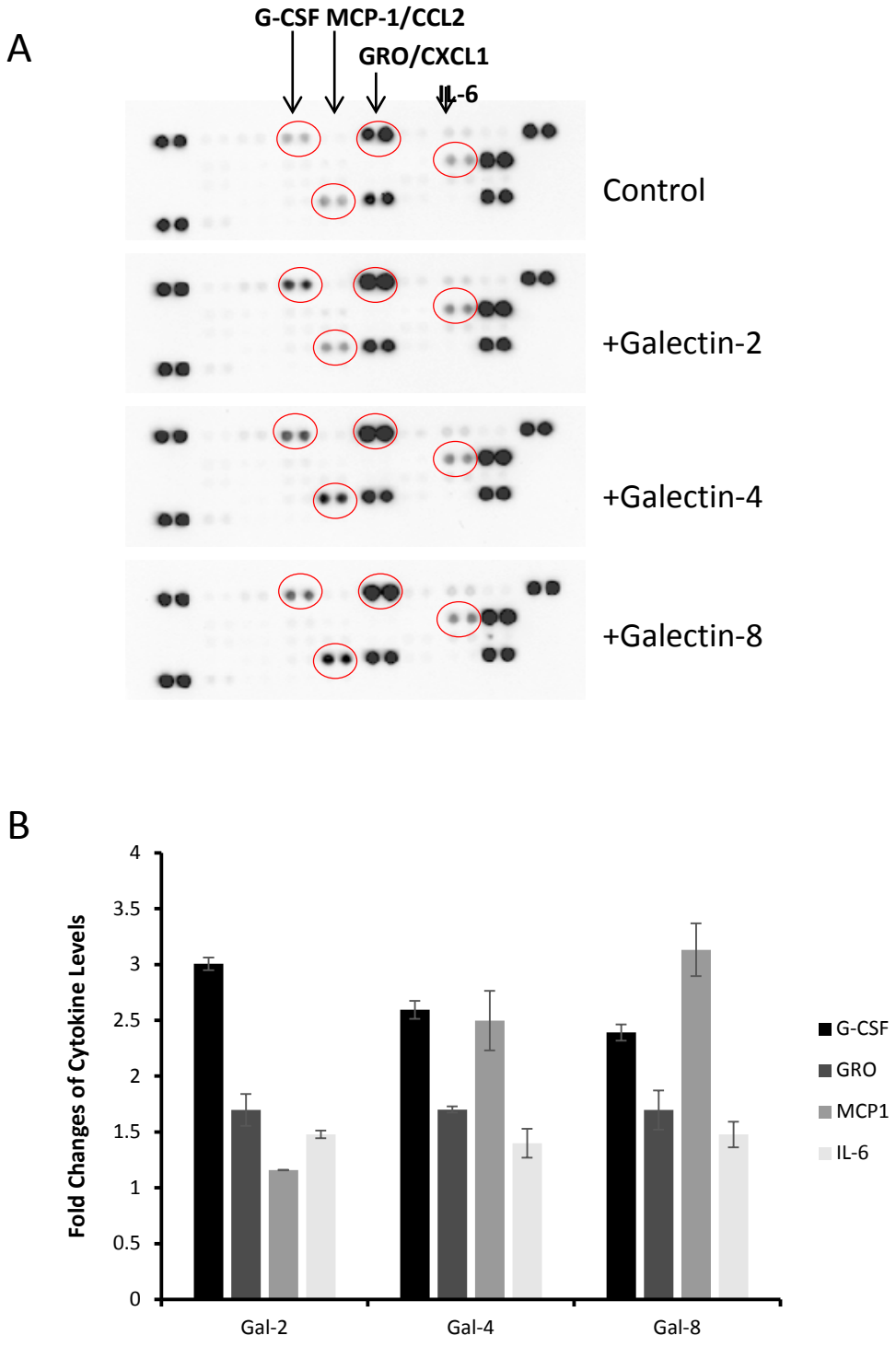



**Fig. 6.1: Galectin-2, -4 or -8 induce secretion of cytokines from HMVEC-Ls after 24 hr culture.** The levels of 36 cytokines in the CM were tested by using Human Cytokine Array Panel Kit and four cytokines (G-CSF, IL-6, GROα and MCP-1) were shown to be increased after

treatment of HMVECs with 1.5μg/ml galectin-2, -4 or -8 for 24 hr (A). Changes in these cytokines were quantified by densitometry scanning of the array (B) (n=2).

After 24-hr treatment with galectins, the levels of four cytokines in the culture medium of HMVECs showed an increase in comparison with the control cells. Galectin-2 treatment appeared to cause an increase of G-CSF (3.00-fold), GROα (1.70-fold), and IL-6 (1.48-fold). Galectin-4 treatment caused an increase of G-CSF (2.59-fold), IL-6 (1.40-fold), GROα (1.70-fold) and MCP-1 (2.99-fold) whereas galectin-8 treatment caused an increase of G-CSF (2.39-fold), IL-6 (1.48-fold), GROα (1.7-fold) and MCP-1 (2.99-fold) (Fig. 6.1).

To investigate whether the G-CSF, IL-6, GROα and MCP-1 secretion induced by galectin-2, -4 or -8 were dose-dependent, confluent HMVEC-Ls cell monolayers were treated with galectin-2, -4 or -8 in different concentrations (BSA control, 0.1, 0.2, 0.5, 1 and 2μg/ml) for 24 hr and the level of G-CSF, IL-6, GROα and MCP-1 in the culture medium was then determined by individual cytokine ELISA.

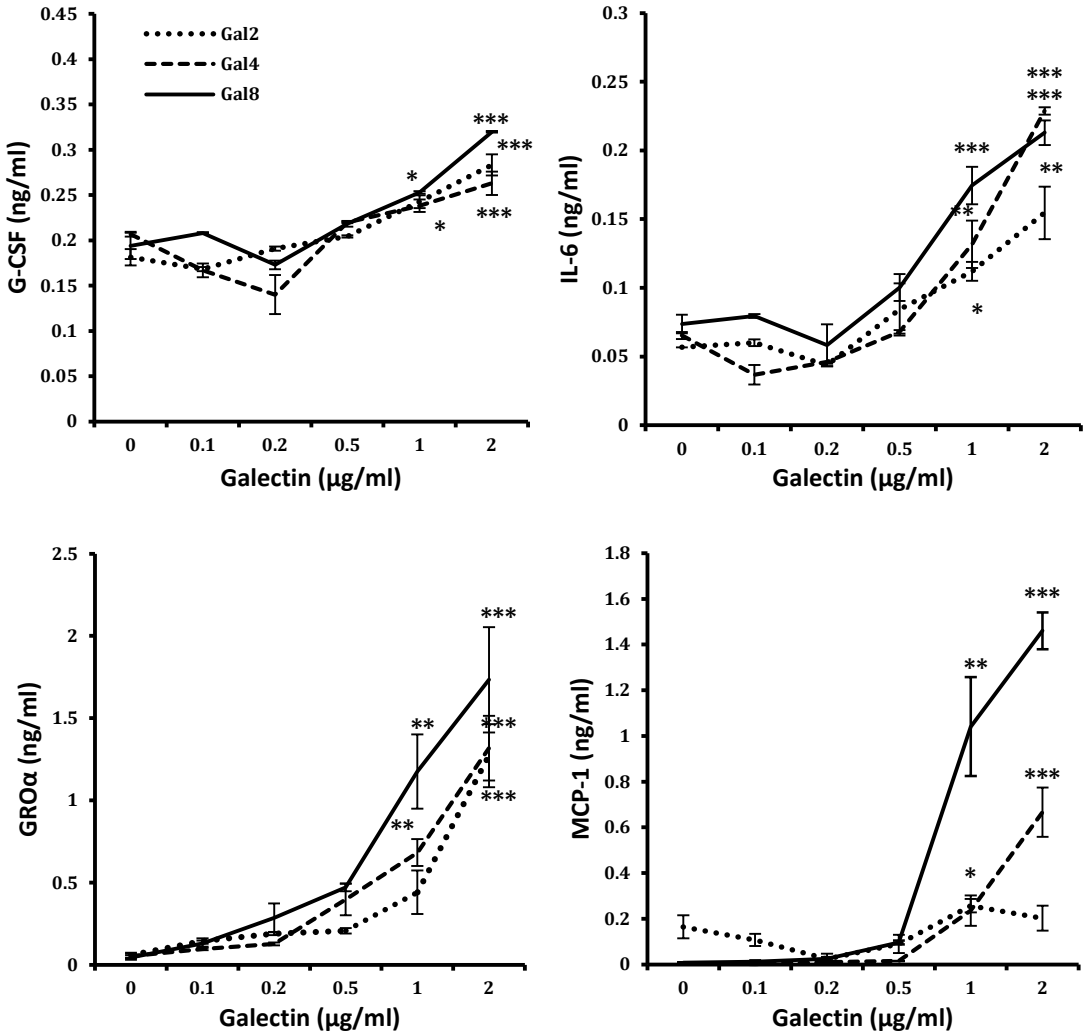



**Fig. 6.2: Galectin-2, -4 and -8 induce dose-dependent secretion of G-CSF, IL-6, GROα and MCP-1 by endothelial cells.** HMVEC-Ls cells were treated with or without various pathological galectin-2, -4 or -8 concentration (BSA control, 0.1, 0.2, 0.5, 1 and 2μg/ml) for 24 hr before the

concentrations of each cytokine in the conditioned medium were determined. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA followed by Bonferroni).

It was found that treatment of the cells with each of these galectins caused a dose-dependent increase of these cytokines (Fig. 6.2 and Table 6.1-6.4).

A 33.8±3.9%, 56.3±6.4% increase of G-CSF was induced by 1 or 2μg/ml galectin-2, 27.1±6.2% by 2μg/ml galectin-4 and 30.4±7.0%, 64.9±0.5% by 1 or 2μg/ml galectin-8 in comparison with the BSA-treated control cells.

A 98.2±12.3%, 175.0±33.5% increase of IL-6 was induced by galectin-2, 101.5±26.0%, 250.8±4.1% by galectin-4 and 138.4±18.5%, 190.4±12.2% by galectin-8 in the culture medium treated after treatment of the cells with 1μg/ml or 2μg/ml galectin-2, -4 or -8 for 24 hr in comparison with the BSA-treated control cells.

A 2091.4±325.6% increase of GROα was induced by 1μg/ml galectin-2, 1159.3±150.5%, 2338.9±363.8% by galectin-4 and 2559.1±513.9%, 3838.6±725.0% by galectin-8 in the culture medium treated with 1μg/ml or 2μg/ml galectin-2, -4 or -8 for 24 hr in comparison with the BSA-treated control cells.

A 4432.3±1278.6%, 12729.1±2111.8% increase in the MCP-1 level was induced by galectin-4 and 13202.0±2816.9%, 18945.4±626.9% by galectin-8 in the culture medium treated with 1μg/ml or 2μg/ml galectin-2, -4 or -8 for 24 hr in comparison with the BSA-treated control cells.

**Table 6.1: Galectin-2, -4 or -8 induce G-CSF secretion from HMVEC-Ls (dose dependent manner)**

| G-CSF | Concentration (μg/ml) | Increase compare to dose 0 (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 0.5 | 12.9 | 1.24 | 0.224 |
| | 1 | 34.1 | 0.61 | 0.002 |
| | 2 | 56.4 | 3.89 | <0.001 |
| **Galectin-4** | 1 | 15.6 | 0.44 | 0.459 |
| | 2 | 27.6 | 3.28 | 0.036 |
| **Galectin-8** | 0.5 | 13.0 | 2.44 | 0.136 |
| | 1 | 31.0 | 1.66 | 0.001 |
| | 2 | 65.7 | 0.69 | <0.001 |

**Table 6.2: Galectin-2, -4 or -8 induce IL-6 secretion from HMVEC-Ls (dose dependent manner)**

| IL-6 | Concentration (μg/ml) | Increase compare to dose 0 (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 0.5 | 48.4 | 33.5 | 0.787 |
| | 1 | 97.3 | 12.3 | 0.043 |
| | 2 | 172.2 | 33.5 | 0.002 |
| **Galectin-4** | 0.5 | 4.12 | 2.0 | 1.000 |
| | 1 | 101.6 | 26.5 | 0.002 |
| | 2 | 250.0 | 4.1 | <0.001 |
| **Galectin-8** | 0.5 | 35.8 | 13.3 | 0.650 |
| | 1 | 136.7 | 18.5 | 0.001 |
| | 2 | 188.8 | 12.2 | <0.001 |

**Table 6.3: Galectin-2, -4 or -8 induce GROα secretion from HMVEC-Ls (dose dependent manner)**

| GROα | Concentration (μg/ml) | Increase compare to dose 0 (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 0.5 | 251.7 | 29.2 | 1.000 |
| | 1 | 649.3 | 225.1 | 0.109 |
| | 2 | 2059.0 | 325.7 | <0.001 |
| **Galectin-4** | 0.5 | 634.8 | 177.3 | 0.172 |
| | 1 | 1159.4 | 150.4 | 0.009 |
| | 2 | 2331.7 | 363.3 | <0.001 |
| **Galectin-8** | 0.5 | 954.8 | 52.3 | 0.611 |
| | 1 | 2528.6 | 505.7 | 0.007 |
| | 2 | 3776.1 | 715.1 | 0.001 |

**Table 6.4: Galectin-2, -4 or -8 induce MCP-1 secretion from HMVEC-Ls (dose dependent manner)**

| MCP-1 | Concentration (μg/ml) | Increase compare to dose 0 (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 0.5 | -45.3 | 24.1 | 1.000 |
| | 1 | 56.2 | 17.6 | 0.832 |
| | 2 | 23.0 | 33.2 | 1.000 |
| **Galectin-4** | 0.5 | 191.8 | 29.1 | 1.000 |
| | 1 | 4434.4 | 1277.3 | 0.064 |
| | 2 | 12730.7 | 2074.8 | <0.001 |
| **Galectin-8** | 0.5 | 1156.7 | 110.3 | 1.000 |
| | 1 | 13474.4 | 2818.6 | 0.001 |
| | 2 | 18945.0 | 1046.2 | <0.001 |

Further experiments were conducted to test if the galectin-mediated secretion of cytokines was time-dependent. A confluent HMVEC-Ls monolayer in a 96-well plate was treated with galectin-2, -4 or -8 (1.5µg/ml) for different times (0, 4, 8, 12, 16, 20 and 24 hr) before the levels of G-CSF, IL-6, GROα and MCP-1 in the culture medium were determined by individual cytokine ELISA.

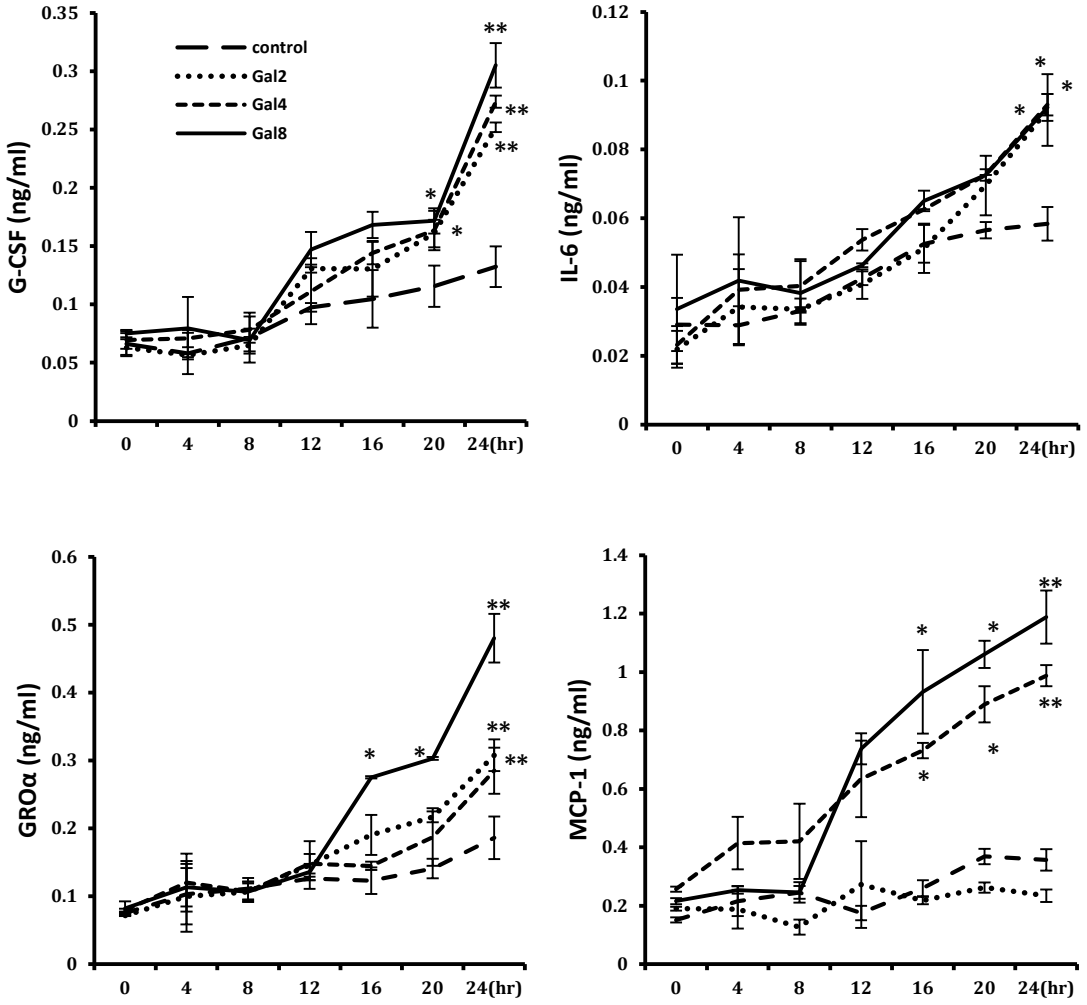



**Fig. 6.3: Galectin-2, -4 and -8 induce time-dependent secretion of G-CSF, IL-6, GROα and MCP-1 by endothelial cells.** HMVEC-Ls cells were treated with 1.5µg/ml galectin-2, -4 or -8 for different times (0 for fresh medium, 4, 8, 12, 16, 20 and 24 hr) before the concentrations of each cytokine in the conditioned medium were determined. The data are expressed as mean ± SD of

triplicate determinations of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA followed by Bonferroni).

In comparison with the medium of BSA-treated cells, increases of 39.2±10.2% at 20hr and 90.5±3.1% at 24 hr levels were seen for galectin-2 , a 41.4±14.6% increase at 20hr and 107.1±3.9% at 24 hr was seen for galectin-4, and a 48.6±9.3% increase at 20hr and 130.7±14.4% at 24 hr of G-CSF was induced by galectin-8 (Fig. 6.3 and Table 6.5-6.8).

In comparison with the BSA-treated cells an increase of 56.9±17.8% at 24 hr was produced by galectin-2, an increase of 60.3±5.4% at 24 hr by galectin-4 and an increase of 58.6±6.5% at 24 hr of IL-6 by galectin-8.

In comparison with the BSA-treated cells an increase of 65.4±16.7% at 24 hr by galectin-2, an increase of 52.7±12.5% at 24 hr by galectin-4 and an increase of 123.6±1.4% at 16hr, 116.4±1.6% at 20hr and 158.2±19.1% at 24 hr of GROα by galectin-8 were observed.

In comparison with the BSA-treated cells an increase of 181.2±10.0% at 16hr, 141.6±16.7% at 20hr and 174.5±10.2% at 24 hr by galectin-4, an increase of 258.5±16.4% at 16hr, 194.4±12.7% at 20hr and 232.5±11.5% at 24 hr of MCP-1 by galectin-8 were observed. Galectin2 did not show any significant effect on MCP1 secretion.

**Table 6.5: Galectin-2, -4 or -8 induce G-CSF secretion from HMVEC-Ls (time-dependent manner)**

| G-CSF | Time point (hr) | Increase compare to BSA treated control (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 16 | 24.7 | 22.9 | 0.57 |
| | 20 | 39.1 | 10.3 | 0.015 |
| | 24 | 90.5 | 3.1 | 0.005 |
| **Galectin-4** | 20 | 37.6 | 9.2 | 0.87 |
| | 24 | 41.4 | 14.6 | 0.014 |
| | 48 | 107.0 | 3.9 | 0.004 |
| **Galectin-8** | 20 | 60.7 | 10.8 | 0.34 |
| | 24 | 48.5 | 9.4 | 0.01 |
| | 48 | 130.6 | 14.5 | 0.004 |

**Table 6.6: Galectin-2, -4 or -8 induce IL-6 secretion from HMVEC-Ls (time-dependent manner)**

| IL-6 | Time point (hr) | Increase compare to BSA treated control (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 20 | 23.0 | 15.4 | 0.28 |
| | 24 | 56.6 | 17.9 | 0.046 |
| **Galectin-4** | 20 | 28.4 | 0 | 0.22 |
| | 24 | 59.3 | 5.3 | 0.044 |
| **Galectin-8** | 20 | 28.4 | 2.9 | 0.22 |
| | 24 | 57.9 | 6.6 | 0.045 |

**Table 6.7: Galectin-2, -4 or -8 induce GROα secretion from HMVEC-Ls (time-dependent manner)**

| GROα | Time point (hr) | Increase compare to BSA treated control (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 20 | 54.0 | 30.0 | 0.10 |
| | 24 | 65.4 | 18.2 | 0.004 |
| **Galectin-4** | 20 | 32.9 | 5.8 | 0.02 |
| | 24 | 53.1 | 12.5 | 0.005 |
| **Galectin-8** | 12 | 7.58 | 9.6 | 0.34 |
| | 16 | 123.59 | 1.3 | 0.012 |
| | 20 | 115.0 | 1.6 | 0.004 |
| | 24 | 158.0 | 19.1 | 0.003 |

**Table 6.8: Galectin-2, -4 or -8 induce GROα secretion from HMVEC-Ls (time-dependent manner)**

| MCP-1 | Time point (hr) | Increase compare to BSA treated control (%) | Standard Deviation | P value |
|---|---|---|---|---|
| **Galectin-2** | 12 | 55.8 | 27.9 | 0.21 |
| | 20 | -28.7 | 4.7 | 0.14 |
| | 24 | -34.4 | 6.1 | 0.10 |
| **Galectin-4** | 12 | 262.2 | 17.5 | 0.32 |
| | 16 | 181.0 | 10.0 | 0.03 |
| | 20 | 141.2 | 16.7 | 0.03 |
| | 24 | 176.5 | 10.2 | 0.03 |
| **Galectin-8** | 12 | 321.1 | 30.3 | 0.22 |
| | 16 | 258.2 | 16.4 | 0.04 |
| | 20 | 187.6 | 12.6 | 0.03 |
| | 24 | 232.6 | 11.5 | 0.02 |

Thus, galectin-2, -4 and -8 at concentrations similar to those in cancer patients induce dose- and time-dependent increase of several cytokines in the vascular endothelium.

### 6.4.2 Investigation of the effect of lactose on galectin-mediated cytokine secretion in HMVEC-Ls

To confirm whether galectin-2, -4 or -8 induce cytokine secretion was mediated through the galectin carbohydrate binding sites, we further assessed the effect of the galectin-binding inhibitor lactose on galectin-mediated cytokine secretion.

First 1.5µg/ml of galectin-2, -4 or -8 were pre-incubated with/without 10µM of lactose for 10 minutes before application to (100µl/well) a 96-well plate with confluent HMVEC-Ls cell monolayers. After 24 hr incubation, the levels of G-CSF, IL-6, GROα and MCP-1 in the conditioned medium were determined by ELISA.

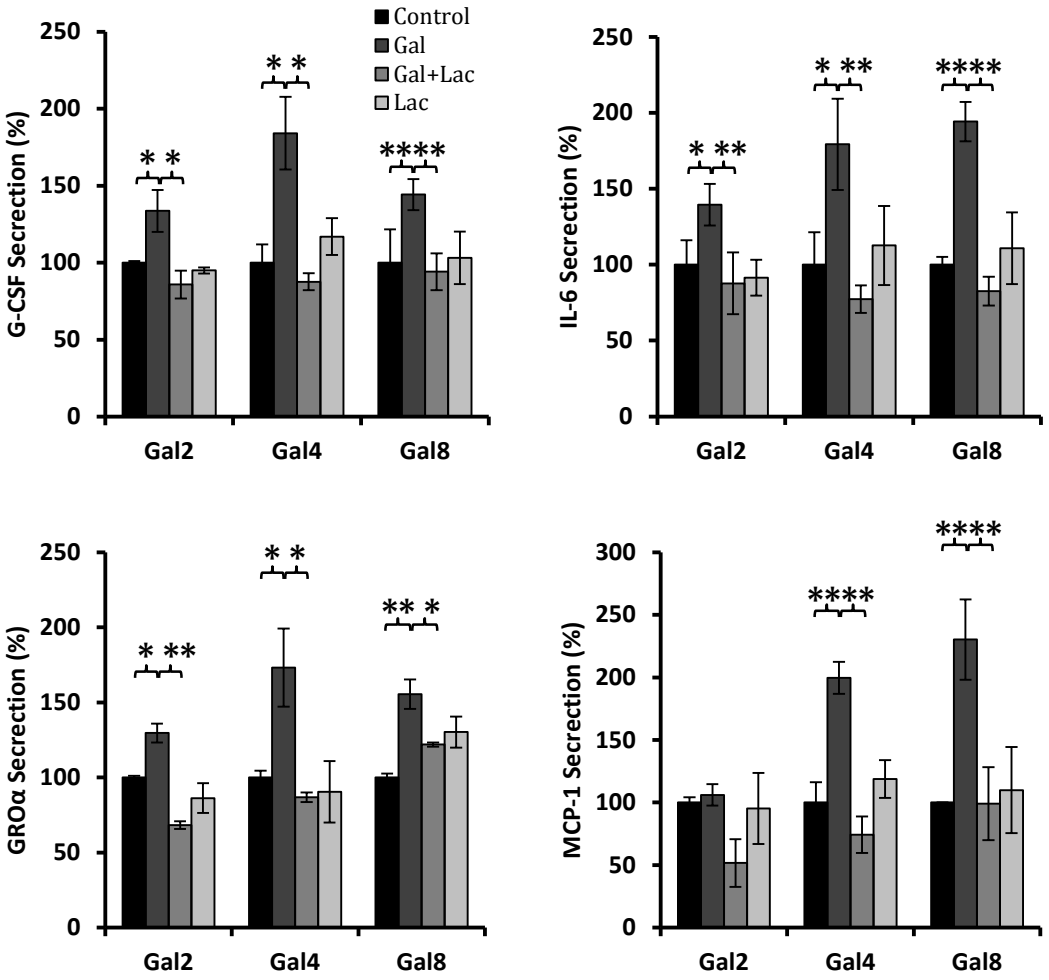



**Fig. 6.4: Galectin-2, -4 or -8 induced G-CSF, IL-6, GROα and MCP-1 secretion from HMVEC-Ls inhibited by the presence of lactose.** The HMVEC-Ls monolayers were treated with 1.5μg/ml galectin-2, -4 or -8 mixed with/without 10μM lactose for 24 hr before the conditioned medium was collected and the level of G-CSF, IL-6, GROα and MCP-1 was determined. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni).

It was found that the increased secretions of G-CSF induced by galectin-2(33.6±13.6%), -4(84.0±33.5%) or -8(44.3±9.9%) are all abolished in the presence of lactose. Similarly, the increased secretions of IL-6 induced by galectin-2(39.4±13.7%),

-4(79.5±30.0%) or -8(94.2±12.9%) are all abolished in the presence of lactose. The increased secretions of GROα induced by galectin-2(29.6±6.2%), -4(73.2±24.6%) are abolished, and the increased secretions of GROα induced by galectin-8(55.5±9.8%) are inhibited (60.4±2.6%) in the presence of lactose. Secretions of MCP-1 induced by galectin-4(99.8±12.8%) were abolished and the increased secretion of MCP-1 induced by galectin-8 (150.0±32.1%) was inhibited (87.6±19.4%) in the presence of lactose (Fig. 6.4).

### 6.4.3 Investigation of effect of galectin-induced cytokine secretion by HMVEC-Ls on cancer cell adhesion

Earlier studies have shown galectin-3 induced secretion of IL-6, G-CSF, GM-CSF and ICAM1 by HMVEC-Ls can enhance cancer cell-endothelial adhesion. As galectin-2, -4 and -8 are also able to induce the secretion of IL-6 and G-CSF, we investigated the effect of galectin-2, -4, -8-mediated secretion of the cytokines by HMVEC-Ls on subsequent cancer cell-endothelial adhesion.

Confluent HMVEC-Ls cell monolayers in each well of a 96-well plate were treated with 1.5μg/ml galectin-2, -4 or -8 for 24 hrs, and BSA was used as negative control. After 24 hr, the culture medium from each well was discarded and 100μl ACA19$^{-}$ human melanoma cells labeled by Calcein AM at a concentration of 1 x $10^5$/ml were added to the HMVEC-Ls monolayer for 1 hr. After they had been washed twice with PBS, the fluorescent intensity in each well which represented the remaining cancer cells was determined.

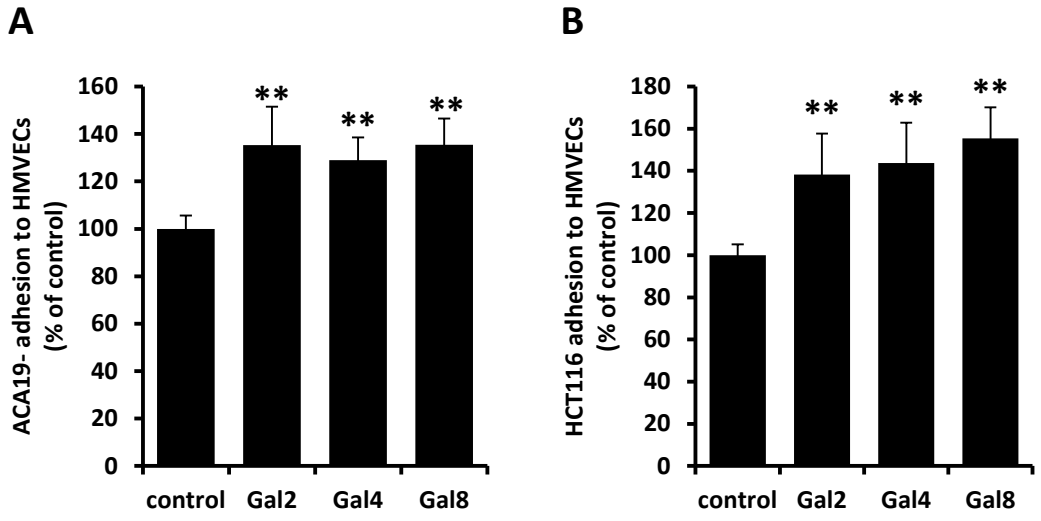



**Fig. 6.5: The presence of galectin-2, -4 or -8 increases ACA19$^-$ (A) or HCT116 cell (B) adhesion to HMVEC-Ls.** The HMVEC-Ls monolayers in each well in a 96-well plate were treated with 1.5µg/ml galectin-2, -4 or -8 for 24 hr before 1 hr adhesion of ACA19$^-$ or HCT116 cell to the treated HMVEC-Ls monolayers was assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni).

It was found that 24 hr pre-treatment of HMVEC-Ls with galectin-2, -4 or -8 resulted in significant increase (35.4±16.1%, 28.9±9.6%, 35.4±11.1%, for galectin-2, -4 and -8 respectively) of ACA19$^-$ cell adhesion (Fig. 6.5 A). Similarly, 24 hr treatment of galectin-2, -4 or -8 resulted in significant increase (38.2±19.6%, 43.7±19.2%, 55.5±14.6%, for galectin-2, -4 and -8 respectively) of HCT116 cell adhesion to HMVEC-Ls (Fig. 6.5 B).

To test whether these galectin effects were associated with the galectin-mediated secretion of cytokines, cell adhesion experiments were further conducted with the conditioned medium obtained from HMVEC-Ls treated for 24 hr with galectin-2, -4 or -8. After 24 hr incubation of confluent HMVEC-Ls monolayers with galectin-2, -4 or -8 (1.5μg/ml), the conditioned medium was collected and used to culture fresh HMVEC-Ls monolayers in each well in 9six-well plate for 1 hr before fresh ACA19$^-$ suspension(100μl/well, 2 x $10^5$/ml) was added in for assessment of cell adhere for 1 hr.

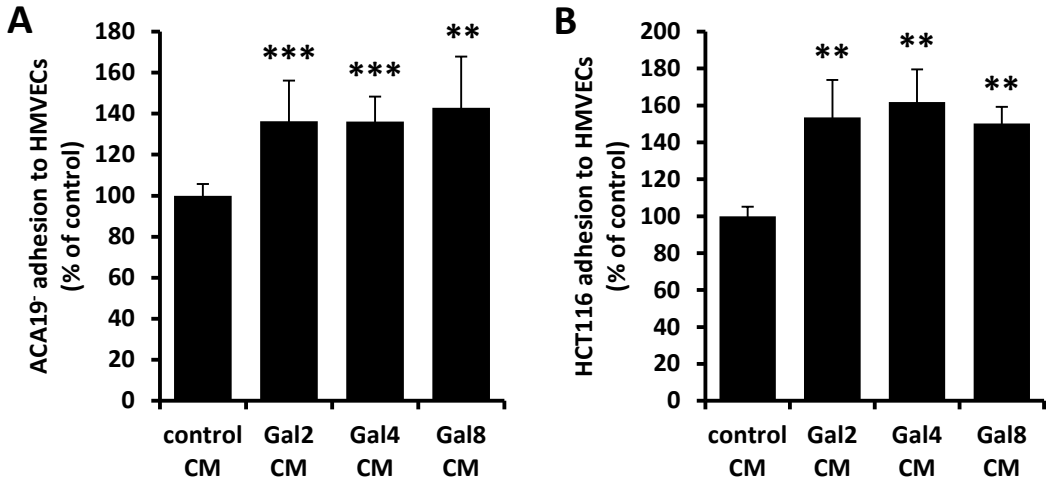



**Fig. 6.6: Galectin-2, -4 or -8 induces secretion of soluble molecules from endothelial cells that cause cancer cell-endothelial adhesion.** The 24 hr treated conditioned medium (CM) from HMVEC-Ls cells treated with or without 1.5μg/ml galectin-2, -4 or -8 were used as conditioned medium to assess adhesion of fresh ACA19$^-$ (A) or HCT116 (B) to fresh HMVEC-Ls monolayer. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (One-way ANOVA followed by Bonferroni).

It was found that the conditioned medium obtained from HMVEC-Ls treated with galectin-2, -4 or -8 (1.5μg/ml) for 24 hr induced a similar increase of subsequent ACA19$^-$ adhesion to fresh HMVEC-Ls monolayers(36.4±19.8%, 36.1±12.2, 42.9±25.0%, for galectin-2, -4 and -8 respectively) (Fig. 6.6 A and Table 6.11). The conditioned medium obtained from HMVEC-Ls treated with galectin-2, -4 or -8 (1.5μg/ml) for 24 hr also showed to induce an increase of subsequent adhesion (53.6±20.2%, 61.9±17.6%, 50.2±9.1%, for galectin-2, -4 and -8 respectively) of HCT116 cells (Fig. 6.6 B).

This result indicates that galectin-2, -4 or -8 induces endothelial secretion of soluble factors, that increase cancer cell-endothelial adhesion

### 6.4.4 Investigation of effect of lactose on galectin-mediated cancer cell adhesion to HMVEC-Ls

To determine whether the galectin-2, -4 or -8 induced increase of cancer cell adhesion was mediated through the galectin carbohydrate binding sites, the galectin binding inhibitor lactose was included in the adhere assay.

In these experiments, galectin-2,-4 or -8, ( 1.5μg/ml) were first mixed with/without 10μM of lactose for 5mins at 37℃ before introduced to confluent HMVEC-Ls cell monolayers for 24 hr at 37℃. BSA was used as negative control. After 24 hr incubation, the medium were discarded and fresh ACA19$^-$ suspension at concentration of 1 x $10^5$/ml was added for 1 hr for assessment of cell adhesion.

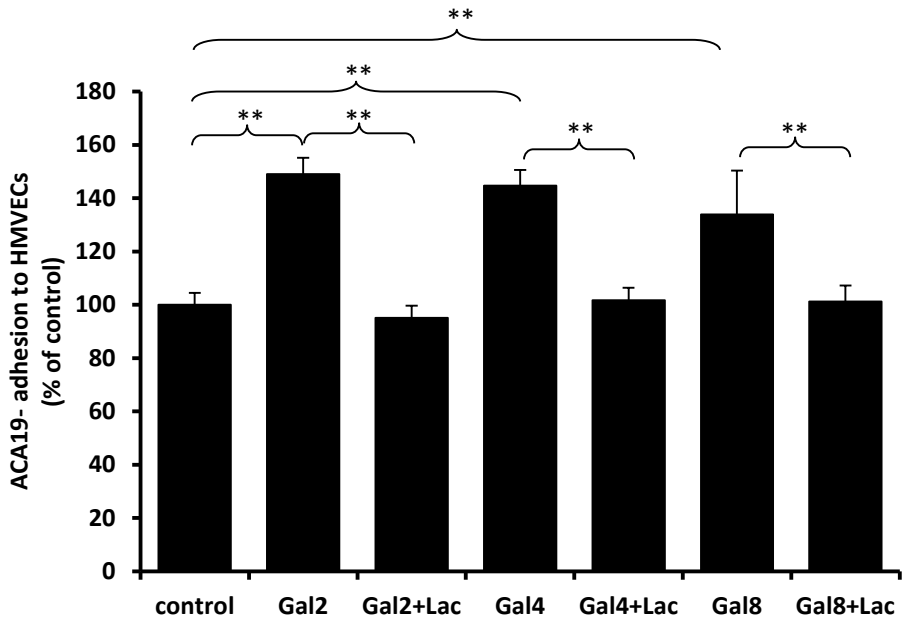



**Fig. 6.7: The presence of lactose abolished galectin-2, -4 or -8 induced increases of ACA19$^-$ cell adhesion to HMVEC-Ls.** The HMVEC-Ls monolayers in each well in 96-well plates were treated with 1.5μg/ml galectin-2, -4 or -8 mixed with 10μM Lactose for 24 hr before 1 hr adhesion of ACA19$^-$ cell to the treated HMVEC-Ls monolayers were assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01 (One-way ANOVA followed by Bonferroni).

It was shown that, the pre-incubation of galectin-2, -4 or -8 with lactose abolished or nearly abolished (100.0±9.34%, 96.1±10.4%, 96.3±17.5%) the adhesion induced by each of the galectins (Fig. 6.7).

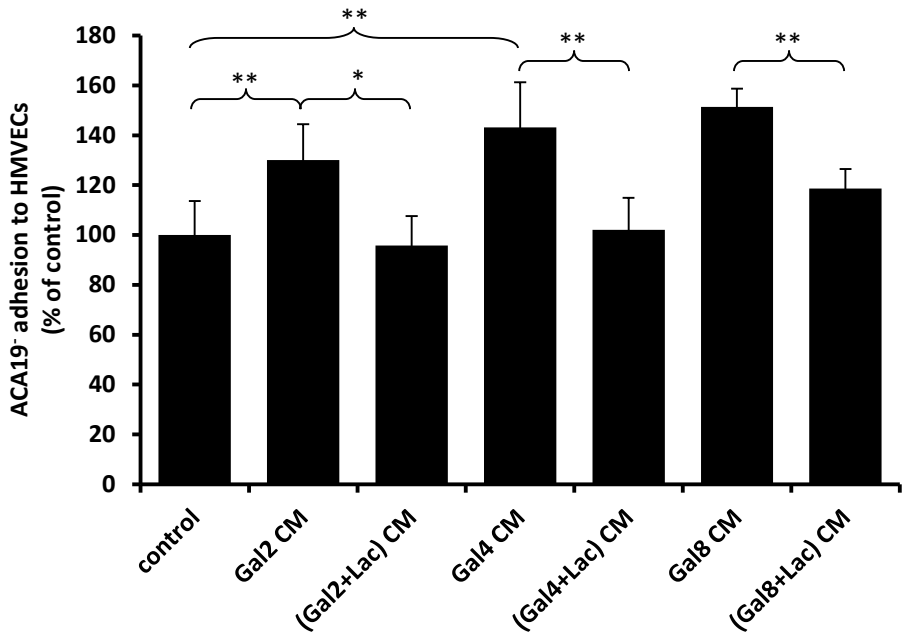



**Fig. 6.8: The conditioned medium obtained from HMVEC-Ls treated with galectin-2, -4 or -8 for 24 hr induced increases of ACA19$^-$ cell adhesion to HMVEC-Ls abolished by the presence of lactose.** The HMVEC-Ls monolayers were treated with 1.5μg/ml galectin-2, -4 or -8 mixed with/without 10μM Lactose for 24 hr before the conditioned medium was collected. The conditioned medium was then used to culture fresh HMVEC-Ls monolayers before 1 hr adhesion of ACA19$^-$ cell suspension to these HMVEC-Ls monolayers. The ACA19$^-$ cells adhere to HMVEC-Ls was assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01 (One-way ANOVA followed by Bonferroni).

Furthermore, 1 hr treatment of fresh HMVEC-Ls with the conditioned medium obtained from HMVEC-Ls treated with galectin-2 for 24 hr induced-increase of ACA19$^-$ adhesion to HMVEC-Ls (30.1±14.4%) was abolished while the increase

induced by galectin-4 or-8 (43.2±18.0%, 51.3±7.4%) were inhibited(95.2±29.5%, 63.8±15.3%) by the presence of lactose (Fig. 6.8).

These results indicate that the presence of lactose can effectively inhibits galectin-2, -4 or -8 induced cancer cell-endothelial adhesions, thus galectin-2, -4 or -8-induced cell adhesion are likely through their carbohydrate binding sites.

### 6.4.5 Investigation of effect of galectin-induced cytokine secretion on galectin-mediated cancer cell-endothelial adhesion

To determine whether the increased secretion of cytokines by HMVEC-Ls in response to galectin-2, -4 or -8 are responsible for the galectin-2, -4 or -8 mediated cancer cell-endothelial adhesion, a combination of anti-cytokine antibodies (antibodies against G-CSF, GROα and IL-6 for galectin-2; antibodies against G-CSF, GROα, IL-6 and MCP-1 for galectin-4 and galectin-8) was included in the adhesion assessment. Confluent HMVEC-Ls cell monolayers in 96 well plates were treated with galectin-2, -4 or -8 (1.5μg/ml) for 24 hrs, and BSA was used as negative control. After 24 hr incubation, the medium in wells were collected. Anti-cytokine antibodies mixture (G-CSF (5ng/ml), IL-6(3ng/ml), GROα (20ng/ml) were added to the Conditioned medium obtained from galectin2-treated HMVEC, G-CSF (5ng/ml), IL-6(3ng/ml), GROα (20ng/ml) and MCP-1(20ng/ml) to galectin-4 or galectin-8 treated conditioned medium before the conditioned medium were used to test ACA19$^{-}$ 1 x $10^5$/ml cell adhesion to fresh HMVEC-Ls.

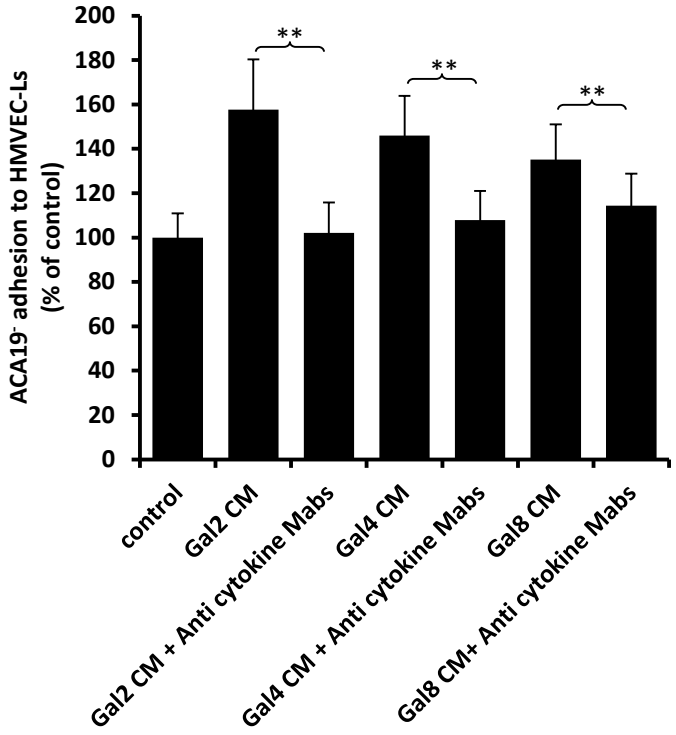



**Fig. 6.9: The presence of anti-cytokine antibodies mixture abolished galectin-2, -4 or -8 treated conditioned medium induced increases of ACA19$^-$ cell adhesion to HMVEC-Ls.** The conditioned medium from HMVEC-Ls treated with 1.5µg/ml galectin-2, -4 or -8 mixed with anti-cytokine antibodies mixture for 1 hr before 1 hr adhesion of ACA19$^-$ cell to the treated HMVEC-Ls monolayers were assessed. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01 (One-way ANOVA followed by Bonferroni).

It was found that, the conditioned medium from galectin-2, -4 or -8 treated HMVECs increased ACA19$^-$ adhesion to fresh HMVEC-Ls (57.6±22.7%, 46.00±18.00%, 35.1±15.9%, respectively). The introduction of antibodies against these cytokines cause inhibition of galectin-2, -4 or -8 induced ACA19$^-$ adhesion to HMVEC-Ls (96.2±23.6%, 82.8±28.5%, 59.1±41.0%, respectively) (Fig. 6.9).

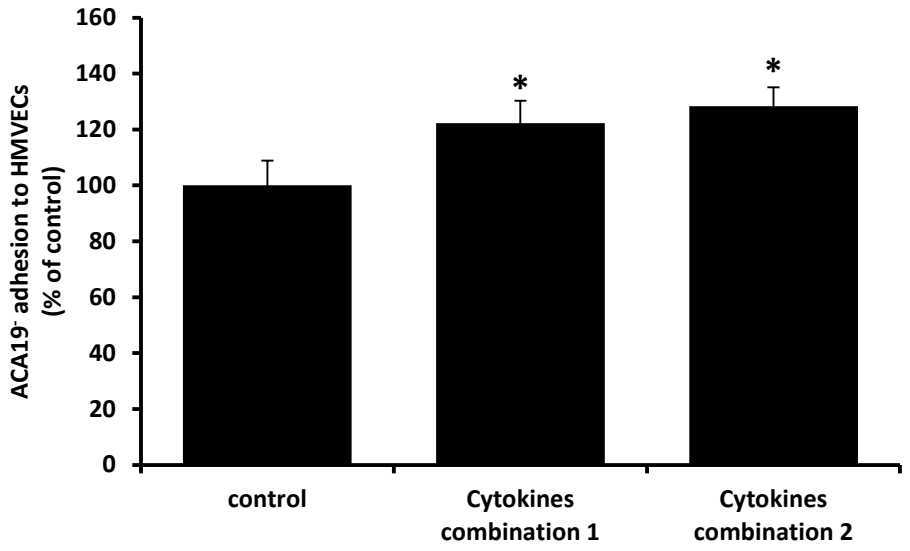



**Fig. 6.10: The presence of conditioned medium form HMVEC-Ls cultured with a combination of recombinant cytokines increases ACA19$^-$ adhesion to HMVEC-Ls.** The combinations of cytokines (Combination 1: G-CSF (0.25ng/ml), IL-6 (0.15ng/ml) and GROα (1ng/ml); Combination 2: (G-CSF (0.25ng/ml), IL-6 (0.15ng/ml), GROα (1ng/ml) and MCP-1 (1ng/ml)) were added to the HMVEC-Ls monolayer culture for 24 hr. The conditioned medium was applied to HMVEC-Ls during 1 hr adhesion of ACA19$^-$ cells to the HMVEC-Ls monolayers. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *$p<0.05$ (one-way ANOVA followed by Bonferroni).

Furthermore, incubation of a combination of three [(G-CSF (0.25ng/ml), IL-6 (0.15ng/ml) and GROα (1ng/ml)] or four [(G-CSF (0.25ng/ml), IL-6 (0.15ng/ml), GROα (1ng/ml) and MCP-1 (1ng/ml)] cytokines with HMVEC-Ls monolayers for 24 hr means the conditioned medium also caused significant increase (22.3±8.1%, 28.4±6.7% respectively) in ACA19$^-$ cell adhesion to the fresh HMVEC-Ls monolayers (Fig. 6.10).

Together these results suggest that the galectin-induced secretion of cytokines is responsible for the observed increase of galectin-2,-4 or 8-mediated cancer cell adhesion.

### 6.4.6 Investigation of effect of galectin-2, -4 or -8 treatment on expression of cell surface adhesion molecules on HMVEC-Ls

To gain an insight into the mechanism of the galectin-2, -4 or -8-induced cytokine-mediated cell adhesion, the expressions of several common cell surface adhesion molecules on HMVEC-Ls were analyzed after treatment of the cells with each galectin. HMVEC-Ls cells were treated with galectin-2, -4 or -8 (1.5μg/ml) for 24 hr before the expressions of endothelial cell surface adhesion molecules (integrin$\alpha_5\beta_1$, CD44, E-selectin, VCAM) were analyzed by flow cytometry.

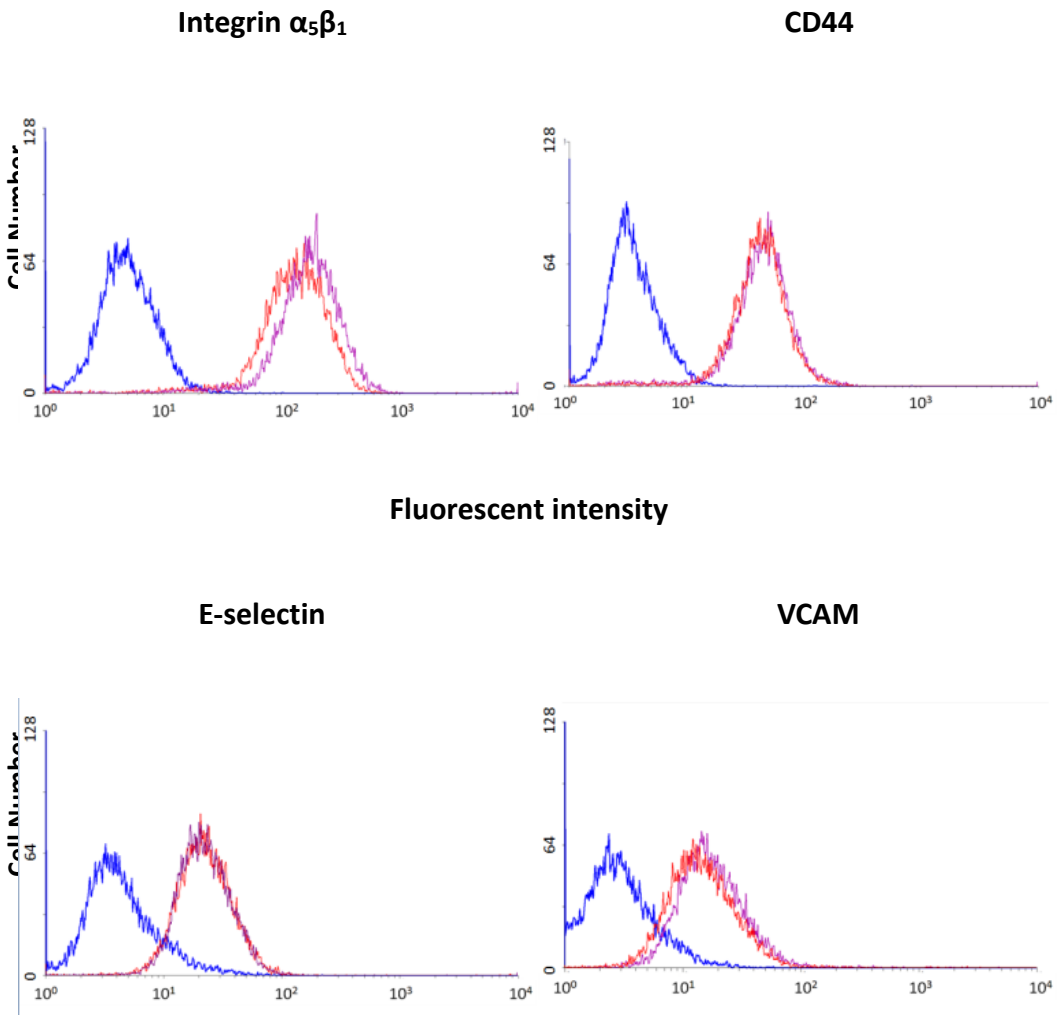



**Fig. 6.11: Galectin-2 induces expression of cell surface integrin$\alpha_5\beta_1$ and VCAM but not CD44 or E-selectin.** The HMVEC-Ls monolayer was treated without (red) or with 1.5µg/ml galectin-2 (purple) for 24 hr before the expressions of HMVEC-Ls cell surface adhesion molecules were analyzed by flow cytometry (n=3). The IgG control is shown in blue. The graph shown is representitive for three independent experiments.

It was found that 24 hr treatment of HMVECs with galectin-2 induces increase of cell surface integrin$\alpha_5\beta_1$ (34.8%) a slight increase of VCAM (9.8%) but not CD44 or E-Selectin (Fig. 6.11).

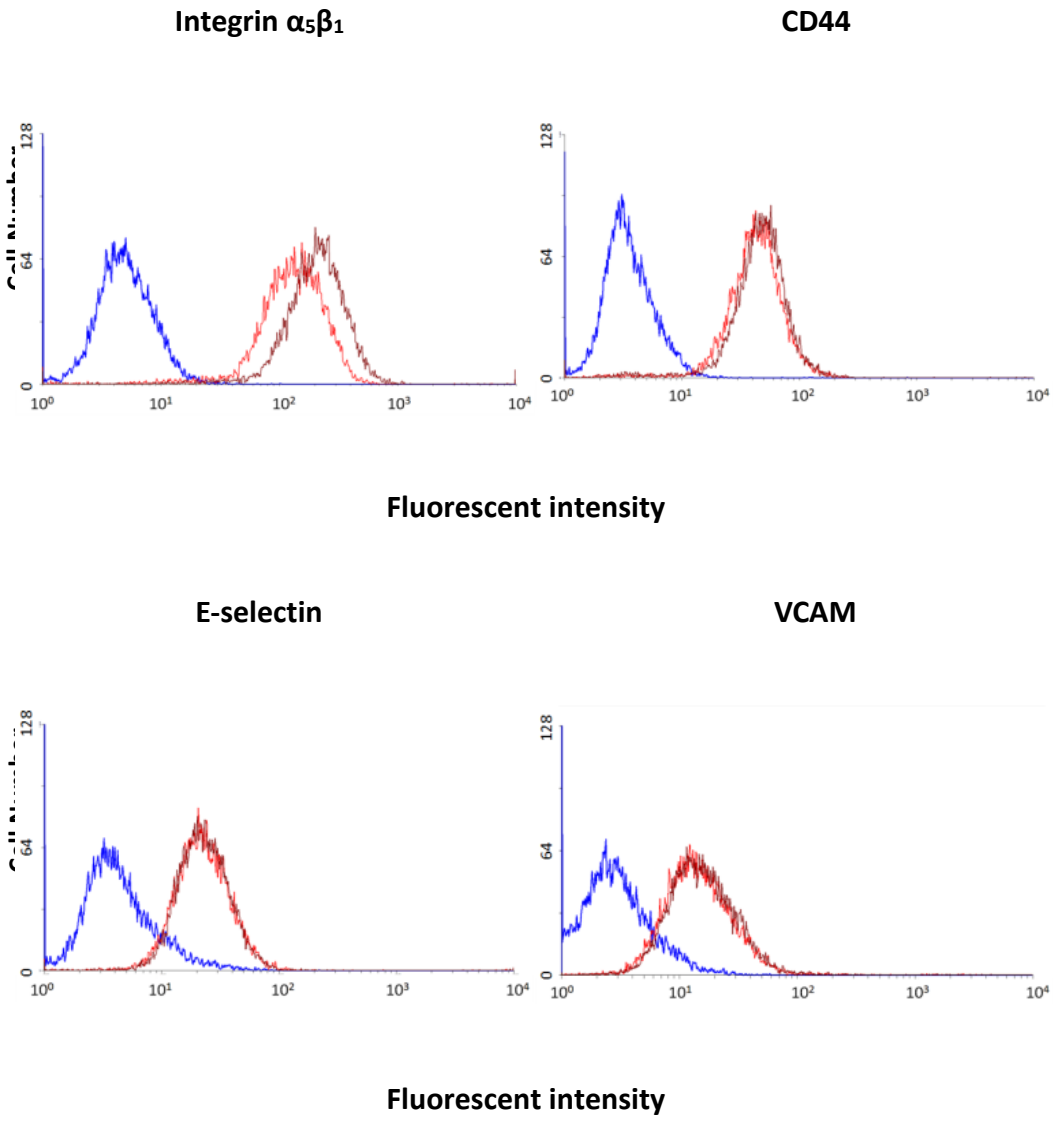



**Fig. 6.12: Galectin-4 induces expression of cell surface integrin$\alpha_5\beta_1$ and CD44 but not VCAM or E-selectin. The** HMVEC-Ls monolayer was treated without (red) or with 1.5µg/ml galectin-4 (brown) for 24 hr before the expressions of HMVEC-Ls cell surface adhesion molecules were analyzed by flow cytometry (n=3). The IgG control is shown in blue. The graph shown is representitive for three independent experiments.

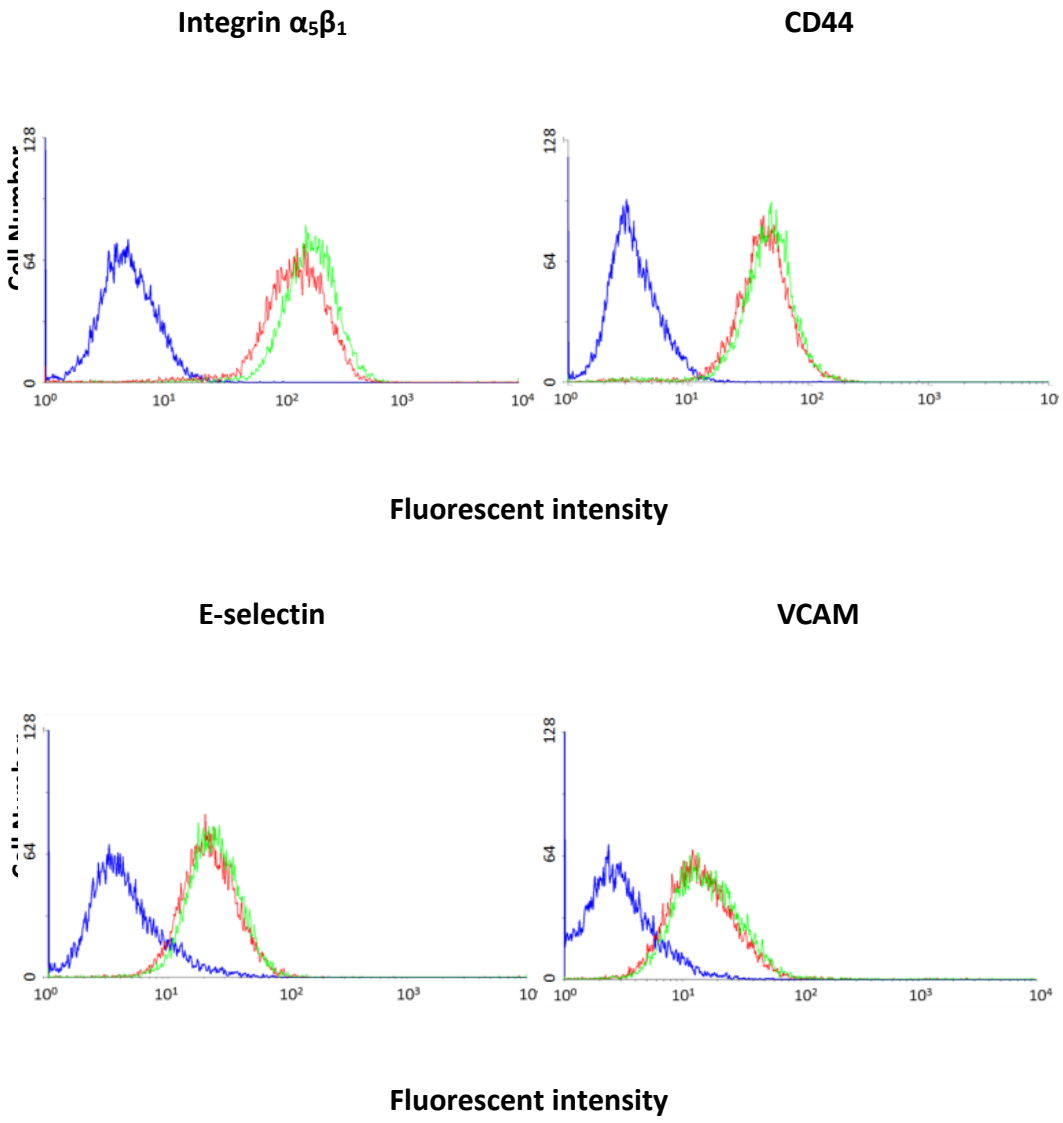



**Fig. 6.13: Galectin-8 induces expression of cell surface integrin$\alpha_5\beta_1$ and CD44 but not VCAM or E-selectin.** The HMVEC-Ls monolayer was treated without (red) or with 1.5µg/ml galectin-8 (green) for 24 hr before the expressions of HMVEC-Ls cell surface adhesion molecules were analyzed by flow cytometry (n=3). The IgG control is shown in blue. The graph shown is representitive for three independent experiments.

It was found that 24 hr treatment of HMVECs with galectin-4 induced increase of integrin$\alpha_5\beta_1$ (41.0%) and CD44 (8.9%) but not E-Selectin or VCAM and galectin-8-treatement increased the expression of integrin$\alpha_5\beta_1$ (32.4%) and CD44 (5.8%) but not E-Selectin or VCAM on the cell surface of HMVEC-Ls (Fig. 6.12, 6.13).

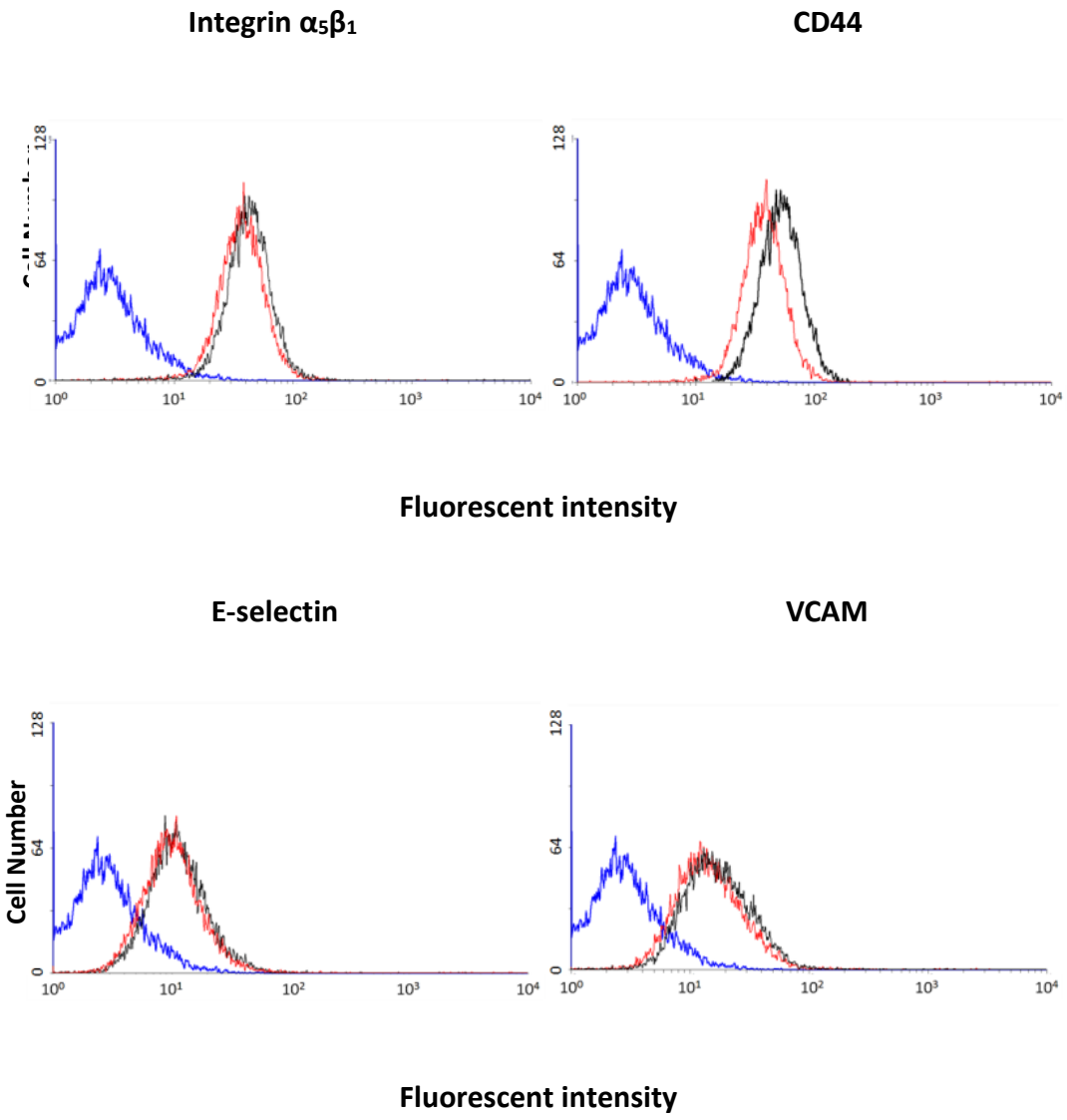


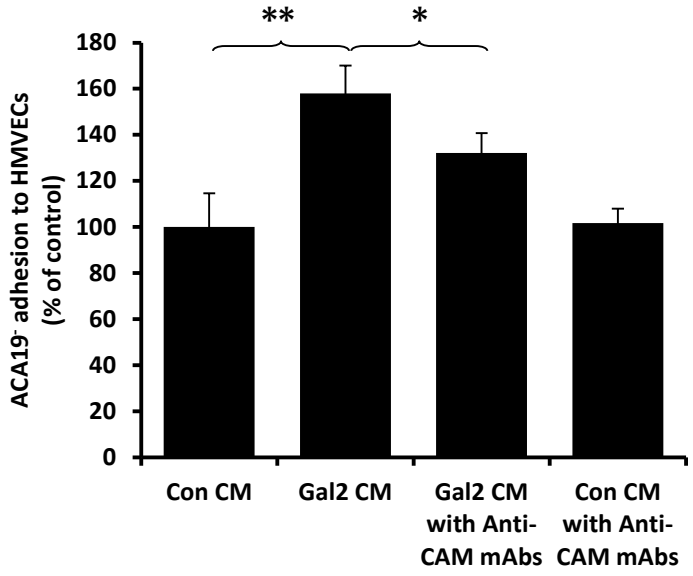

**Fig. 6.14: A combination of cytokines (G-CSF, IL-6, GROα and MCP-1) induces expression of cell surface integrin$\alpha_5\beta_1$, CD44, VCAM but not E-selectin.** The HMVEC-Ls monolayer was treated without (red) or with 1.5µg/ml galectin-8 (black) for 24 hr before the expressions of HMVEC-Ls

cell surface adhesion molecules were analyzed by flow cytometry (n=3). The IgG control is shown in blue. The graph shown is representitive for three independent experiments.

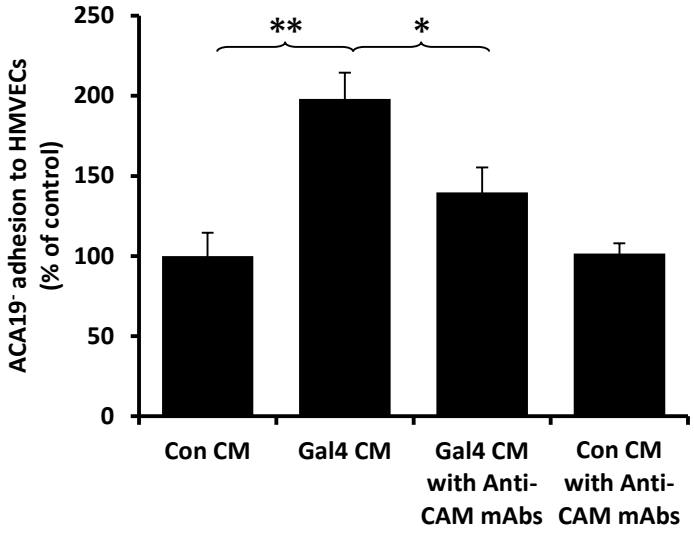
Treatment of HMVEC-Ls cells with a combination of recombinant cytokines (G-CSF (0.25ng/ml), IL-6 (0.15ng/ml), GROα (1ng/ml), MCP-1 (1ng/ml)) induced increase of integrin$\alpha_5\beta_1$ (42.8%), CD44 (11.6%), VCAM (8.7%) but not E-selectin on the cell surface of HMVEC-Ls (Fig. 6.14).

To identify the role of cell surface adhesion molecules in galectin-mediated cancer cell-endothelial adhesion, antibodies against the cell surface adhesion molecules (CAM) were included in the assessment. In this experiment confluent HMVEC-Ls cell monolayers were treated with galectin-2, -4 or -8 (1.5µg/ml) for 24 hrs, and BSA was used as negative control. After 24 hr incubation, the conditioned medium was collected. Anti-CAM antibody mixture [(integrin$\alpha_5\beta_1$ (10µg/ml) and VCAM (10µg/ml) for galectin-2, integrin$\alpha_5\beta_1$ (10µg/ml) and CD44 (10µg/ml) for galectin-4 and -8)] was added to the conditioned medium before assessment of subsequent ACA19$^-$ cell adhesion to fresh HMVEC-Ls.

A


**
*
180
160
140
120
100
80
60
40
20
0
ACA19- adhesion to HMVECs (% of control)
Con CM
Gal2 CM
Gal2 CM with Anti-CAM mAbs
Con CM with Anti-CAM mAbs

B


**
*
250
200
150
100
50
0
ACA19- adhesion to HMVECs (% of control)
Con CM
Gal4 CM
Gal4 CM with Anti-CAM mAbs
Con CM with Anti-CAM mAbs

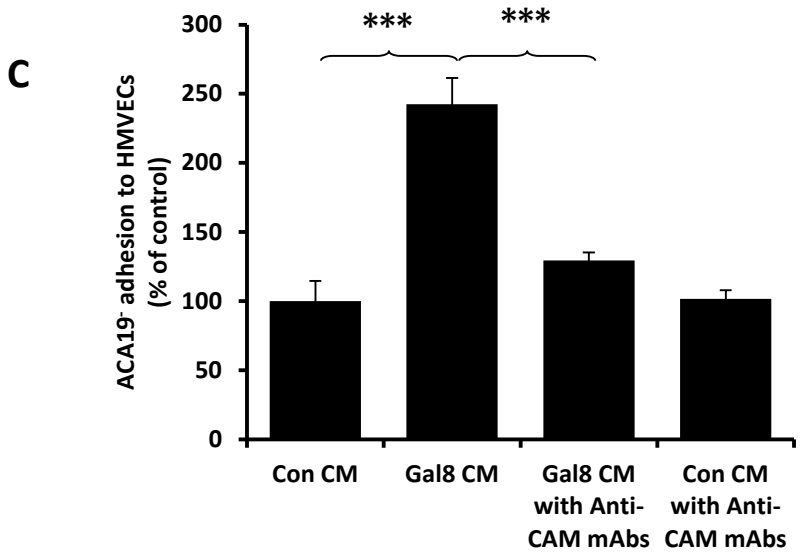



**Fig. 6.15: The presence of antibodies against the cell surface adhesion molecules (CEM) inhibits galectin-2 (A), -4 (B) or -8 (C) mediated cancer cell adhesion.** The conditioned medium from HMVEC-Ls treated with 1.5µg/ml galectin-2, -4 or -8 were mixed with anti-CAM antibody mixture for 1 hr before use as culture medium for assessment of $ACA19^-$ cell adhesion to the treated HMVEC-Ls monolayers. The data are expressed as mean ± SD of triplicate determinations of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA followed by Bonferroni).

It was found that the conditioned medium from galectin-2-treated HMVECs increased $ACA19^-$ adhesion to HMVEC-Ls (58.0 ± 12.0%) and this effect was inhibited (44.6 ± 14.8%) by the presence of the anti-CAM antibodies. Similarly, the conditioned medium from galectin-4-treated HMVECs increased $ACA19^-$ adhesion to HMVEC-Ls (97.9% ± 16.5%), an effect that was inhibited (59.3 ± 15.8%) by the presence of anti-CAM antibodies whereas the conditioned medium from galectin-8-

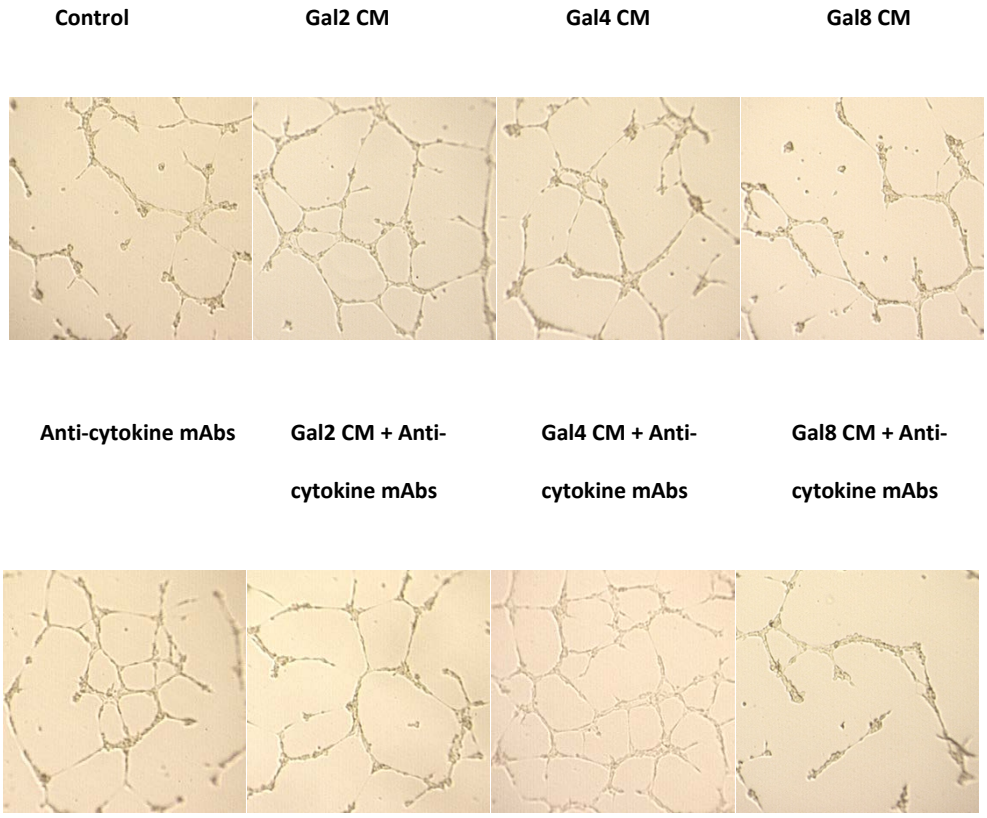
treated HMVECs increased ACA19$^-$ adhesion to HMVEC-Ls (142.4 ± 19.1%) and was inhibited (79.4 ± 4.1%) by the presence of anti-CAM antibodies (Fig. 6.15).

### 6.4.7 Investigation of galectin-2, -4 or -8-mediated cytokine secretion on endothelial tube formation in angiogenesis

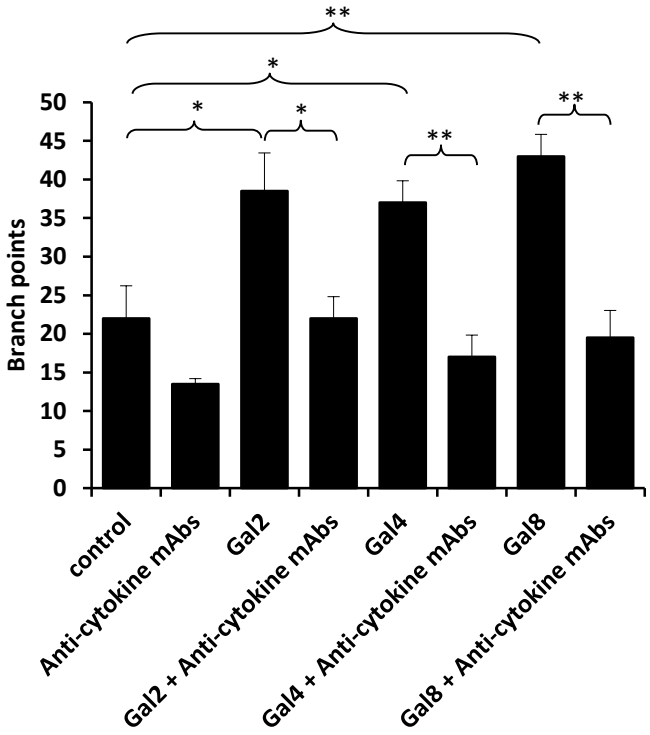
Since our earlier study has shown that galectin-3-induced cytokine secretion promotes endothelial cell tube formation we assessed whether the secretion of cytokines induced by galectin-2, -4 or -8 could also influence the ability of endothelial cells to form microtubules in angiogenesis.

To test this possibility, the conditioned medium from 24 hr galectin-2, -4 or -8 (1.5µg/ml)-treated HMVEC-Ls was collected and used as culture medium to culture HUVECs on matrix. It was found that HUVEC formed significantly more tubule structures when cultured in the conditioned medium from galectin-2 -4 or -8-treated HMVECs than the HUVEC cells cultured in the conditioned medium from BSA-treated HMVEC-Ls.

**A**


Control
Gal2 CM
Gal4 CM
Gal8 CM
Anti-cytokine mAbs
Gal2 CM + Anti-cytokine mAbs
Gal4 CM + Anti-cytokine mAbs
Gal8 CM + Anti-cytokine mAbs

**B**


Branch points
0
5
10
15
20
25
30
35
40
45
50
*
*
*
**
**
**
control
Anti-cytokine mAbs
Gal2
Gal2 + Anti-cytokine mAbs
Gal4
Gal4 + Anti-cytokine mAbs
Gal8
Gal8 + Anti-cytokine mAbs

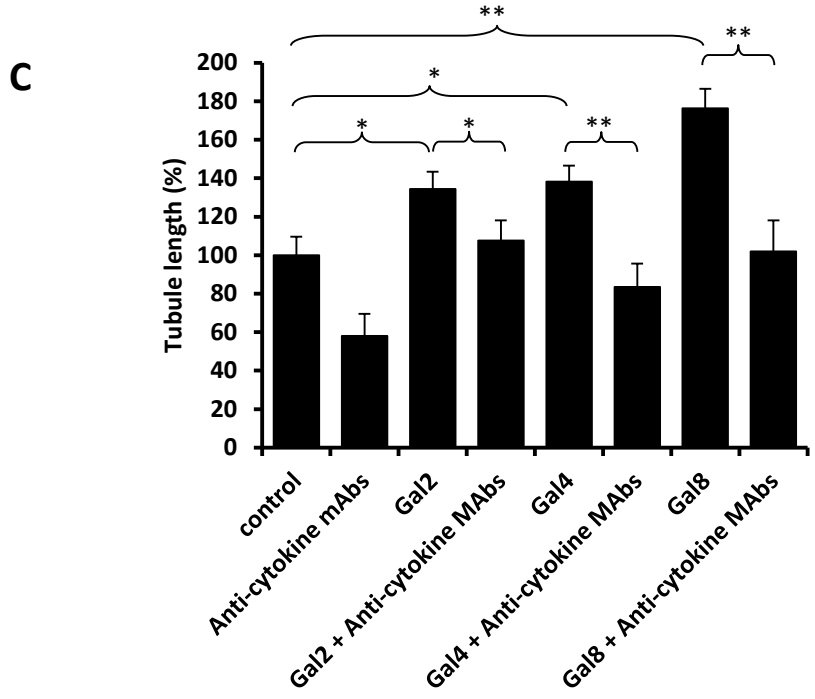



**Fig. 6.16: Galectin-2, -4 or -8-induced cytokine secretion promotes endothelial tubule formation.** HUVEC cells were cultured on top of matrix proteins in the conditioned medium (CM) obtained from HMVEC-Ls treated with BSA or galectin-2, -4 or -8(1.5μg/ml) for 24 hr, with or without introduction to the conditioned medium of a combination of neutralizing antibodies against G-CSF (5μg/ml), IL-6 (3μg/ml), GROα (20μg/ml) and MCP-1(20μg/ml) for 24 hr at 37℃. Representative images are shown in (A). The tubule length (B) and branch points (C) were quantified. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni).

Both the tubule length (34.5±9.0%, 38.3±8.3%, 76.4±10.1% respectively) and branch points (75.0±15.9%, 68.2±9.1%, 95.5±9.1% respectively) were observed to be increased by galectin-2, -4 and -8. The addition of four anti-cytokine antibodies (G-CSF (5μg/ml), IL-6 (3μg/ml), GROα (20μg/ml) and MCP-1 (20μg/ml)) to the conditioned medium before it was applied to the 24 hr HUVEC culture caused

inhibition of tubule length (77.7±22.2%, 100.0±21.3%, 97.5±20.7% respectively) and branch points (100.0±10.0%, 100.0±18.3%, 100.0±9.1% respectively) induced by the conditioned medium from galectin-2, -4 and -8-treated HMVECs (Fig. 6.16).

### 6.4.8 Investigation of effect of injection galectin-2, -4 and/or -8-induced cytokine secretion in mice

Since our earlier study has shown that the injection of galectin-3 induced cytokine secretion in mice, we assessed whether the secretion of cytokines can also be induced by galectin-2, -4 or -8.

First 5µg/mouse galectin-2, -4 or -8, equating approximately to circulating galectin concentrations seen in cancer patients with metastasis (321), was injected intravenously into the animals' tail vein.

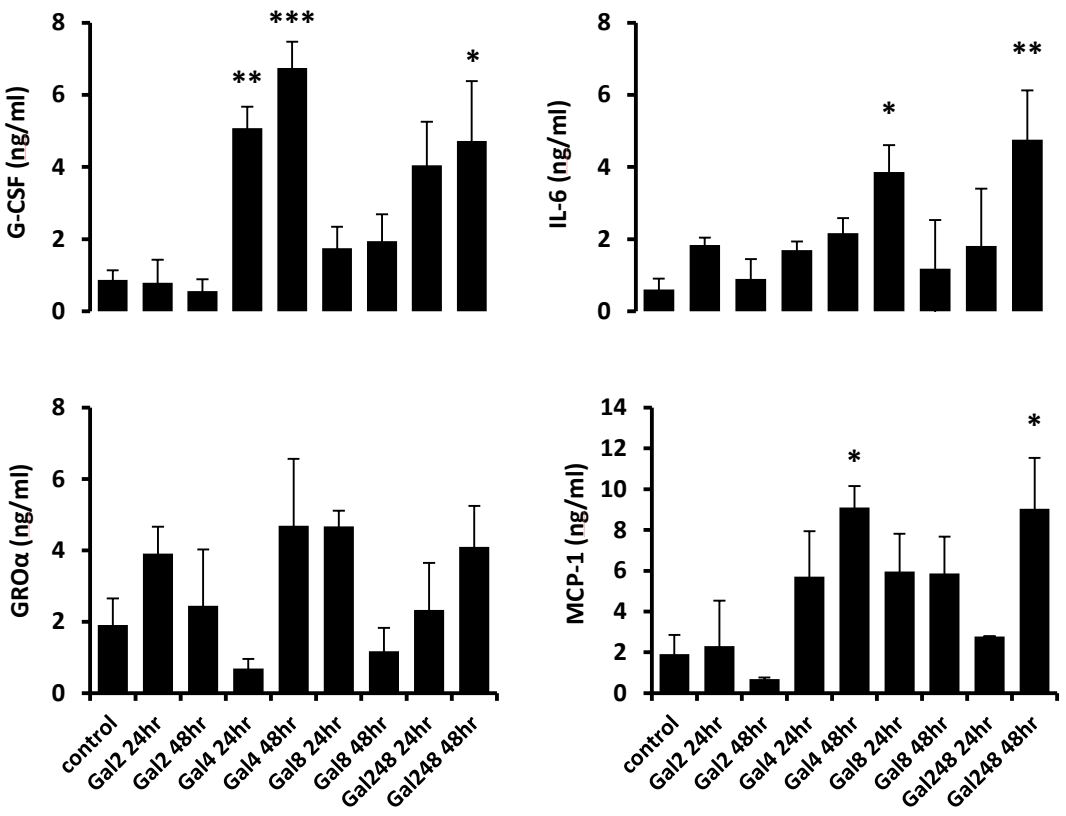



**Fig. 6.17: *In vivo* experiments showing that galectins induce cytokine secretion.** The graphs show the *in vivo* cytokine secretion induction effect of tail vein injection of 5μg galectin-2, -4, -8, individually or in combination (5μg each) on serum levels of the cytokines/chemokines at 0, 24 and 48 hr in mice, three mice in each group. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni).

A 490.7±68.6% and 683.7±83.7% increase of serum G-CSF was seen at 24 and 48 hr for galectin-4 (Fig. 6.17). A 375.9±54.9% increase of MCP-1/CCL2 was also observed after 48 hr for galectin-4. Galectin-8 injection caused a 543.3±123.3% increase of serum IL-6 after 48 hr. Galectin-2 injection did not show any significant effect on serum levels of any of these cytokines/chemokines, however. Serum GROα/CXCL1 levels were not affected by injection of any of these galectins. Injection

of a combination of 5 µg/mouse of each galectin-2, -4 and -8 increased serum G-CSF (448.8±191.9%), IL-6 (691.7±228.3%) and MCP-1 (373.3±130.4%) after 48 hr and the increase of each of these cytokines/chemokines was equivalent to that produced by the most influential galectin member (galectin-4) when injected individually (Fig. 6.17).

These results provide evidence of a direct impact of circulating galectins on secretion of these cytokines *in vivo*. They also indicate that these galectin members may use the same endothelial receptors to exert their effects on endothelial secretion of these cytokines/chemokines, thus showing lack of an additive effect when they are present in combination.

### 6.4.9 Investigation of the relationship between serum levels of galectins and cytokines/chemokines in breast and colon cancer patients

To see whether the relationship between these galectins and cytokines/chemokines observed *in vitro* and in mice occurred in cancer patients, serum levels of circulating galectin-2, -4 and -8 and G-CSF, IL-6, GROα/CXCL2, and MCP-1/CCL1 were analyzed in breast and colon cancer patients (Table 6.9).

**Table 6.9: Simple and multiple regression analysis of serum galectin-2, -4 and -8 and GCS-F, IL-6, GROα and MCP-1 levels in human breast and colon cancer patients**

| | Simple regression analysis | | | | | | | | Multiple regression analysis | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G-CSF | | IL-6 | | GROα/CXCL-1 | | MCP1/CCL-2 | | G-CSF | | IL-6 | | GROα/CXCL-1 | | MCP1/CCL-2 | |
| | Beta (95%CI) | *p* | Beta (95%CI) | *p* | Beta (95%CI) | *p* | Beta (95%CI) | *p* | Beta (95%CI) | *p* | Beta (95%CI) | *p* | Beta (95%CI) | *p* | Beta (95%CI) | *p* |
| **Breast cancer (n=40)** | | | | | | | | | | | | | | | | |
| Gal-2 | 16.02 (13.42-18.62 | <0.001 | 4.56 (3.84-5.27) | <0.001 | -0.002 (-0.007-0.004) | 0.59 | 0.000 (-0.001-0.001) | 0.71 | 15.97 (12.56-19.39) | <0.001 | 4.56 (3.62-5.51) | <0.001 | -0.0025 (-0.010-0.005) | 0.51 | -0.0002 (-0.002-0.001) | 0.75 |
| Gal-4 | 16.28 (7.93-24.62) | <0.001 | 4.63 (2.28-6.99) | <0.001 | -0.002 (-0.012-0.007) | 0.61 | 0.001 (-0.002-0.003) | 0.60 | -2.87 (-8.53-2.79) | 0.31 | -0.789 (-2.36-0.78) | 0.32 | -0.0029 (-0.015-0.001) | 0.64 | 0.0002 (-0.002-0.003) | 0.88 |
| Gal-8 | 19.96 (8.32-31.60) | 0.001 | 5.55 (2.24-8.86) | 0.002 | 0.008 (-0.005-0.020) | 0.22 | 0.002 (-0.001-0.005) | 0.13 | 6.162 (-0.269-12.59) | 0.06 | 1.59 (-0.19-3.37) | 0.079 | 0.012 (-0.003-0.026) | 0.11 | 0.002 (-0.001-0.005) | 0.16 |
| F test | | NA | | NA | | NA | | NA | | <0.001 | | <0.001 | | 0.40 | | 0.50 |
| **Colon cancer (n=50)** | | | | | | | | | | | | | | | | |
| Gal-2 | 1.64 (1.28-2.01) | <0.001 | 0.13 (0.09-0.17) | <0.001 | 0.00 (-0.011-0.11) | 0.96 | 0.001 (0.003-0.008) | <0.001 | 1.45 (1.08-1.82) | <0.001 | 0.11 (0.064-0.15) | <0.001 | -0.000 -0.012-0.12) | 0.99 | 0.005 (0.0024 -0.007) | <0.001 |
| Gal-4 | 1.34 (0.50-2.18) | 0.002 | 0.14 (0.04-0.2) | 0.003 | -0.002 (-0.019-0.015) | 0.82 | 0.003 (-0.001-0.008) | 0.15 | 0.56 (0.006-1.12) | 0.048 | 0.064 (-0.002-0.13) | 0.056 | -0.002 (-0.02-0.16) | 0.82 | 0.0002 (-0.003-0.004) | 0.91 |
| Gal-8 | 2.07 (0.39-3.76) | 0.017 | 0.20 (0.04-0.36) | 0.017 | 0.002 (-0.031-0.035) | 0.90 | 0.13 (0.005-0.020) | 0.002 | 1.04 (0.005-2.10) | 0.051 | 0.114 (-0.01-0.24) | 0.069 | 0.002 (-0.031-0.037) | 0.88 | 0.0095 (0.002-0.16) | <0.001 |
| F test | | NA | | NA | | NA | | NA | | <0.001 | | <0.001 | | 0.99 | | <0.001 |

Serum concentrations of G-CSF, IL-6, GROα/CXCL-1 and MCP-1/CCL-2 in 50 colorectal cancer patients with and without clinically detectable liver metastasis as well as 40 breast cancer patients were determined by galectin-2, -4 or -8 ELISA. The relationship between serum galectin-2, -4, -8 and each of those cytokines was analyzed by simple regression analysis and multiple regressions.

Simple regression analysis showed significant correlation of G-CSF and IL-6 levels, but not GROα and MCP-1, with each of the galectins in both breast and colon cancer patients (Table 6.9). Multiple regression analysis demonstrated significant correlation of G-CSF ($p<0.001$) and IL-6 level ($p<0.001$) with galectin-2 in breast and colon cancer. G-CSF and IL-6 levels were significantly ($p=0.048$) or nearly significantly ($p=0.056$) correlated with galectin-4 in colon cancer but not in breast cancer. Both G-CSF and IL-6 showed close to significant correlation with galectin-8 in breast ($p=0.06$ and 0.079) and colon cancer ($p=0.051$ and 0.069). These observations provide further support for the role of these galectins in secretion of these cytokines in the presence of cancer. It should be mentioned that as circulating galectin levels are correlated with each other in the presence of cancer, the multiple regression analysis is likely to identify only the significance of the strongest association but mask other weaker associations between the galectins and cytokines/chemokines.

## 6.5 Discussion

The results in this chapter show that galectin-2, -4 and -8, at pathological concentrations found in the bloodstream of cancer patients, induce secretion of G-CSF, IL-6, GROα/CXCL1 and MCP-1/CCL2 from the vascular endothelium *in vitro* and in mice. Such a relationship between circulating galectin-2, -4, -8 and G-CSF, IL-6 and MCP-1/CCL2 was also observed in the sera of breast and colon cancer patients. The galectin-induced secretion of these cytokines/chemokines was shown to enhance the expression of endothelial cell surface adhesion molecules, which causes increased cancer cell-endothelial adhesion, and also increases endothelial tubule formation, a component of angiogenesis. Thus, the increased circulation of galectin-2, -4 and -8 found in cancer likely makes a very important contribution to the elevated circulating concentrations of these cytokines/chemokines that are frequently observed in cancer patients.

GROα/CXCL1 and MCP-1/CCL2 are chemoattractant cytokines and, like many other chemokines, promote cancer progression and metastasis (424-426) through several mechanisms such as increased angiogenesis, activation of tumor-specific immune responses to weaken the host anti-tumor immunity, stimulation of tumor cell proliferation and metastasis(124, 424).

Interaction of GROα/CXCL1 with its receptor CXCR2 promotes cancer cell invasion and migration by activation of cellular AKT/NF-kB signaling (427). Administration of anti-GROα/CXCL1 antibodies inhibits tumor formation and angiogenesis *in vitro* and in mice (428). Suppression of GROα/CXCL1 expression in

human colon cancer LS174T cells by shRNA before cell inoculation into the spleens of nude mice almost completely prevents liver metastasis in comparison with those inoculated with parental LS174T cells(429). Higher serum concentrations of GRO/CXCL1 are seen in cancer and, like IL-6, it promotes colonization of circulating tumor cells to their origin (self-seeding) thus accelerating the growth and angiogenesis of human breast, colon and melanoma tumors(398). Recently, GROα/CXCL1 has been reported to be at the center of chemo-resistance triggered by chemotherapeutic agents of breast cancer by helping the tumor cells to recruit pro-survival factor S100A8/9 (425).

MCP-1/CCL2, through interaction with its receptor CCR2 in cancer cells, increases cancer cell invasion and migration by activation of protein kinase C and protein tyrosine phosphorylation (430, 431). Inhibition of MCP-1/CCL2 biosynthesis by MCP-1/CCL2 inhibitor *bindarit* inhibits cancer cell proliferation and migration *in vitro* and significantly impairs metastasis of prostate cancer in mouse xenografts (432). More lung metastases were formed in MCP-1(-/-) mice than in wild-type mice when 4T1 breast cancer cells were transplanted into the mammary pads (433). MCP-1/CCL2 concentrations are frequently elevated in cancer patients(434) and, like IL-6, it is involved in driving the “stemness” of tumor-initiating cells(434).

Thus, IL-6, G-CSF, GROα/CXCL1 and MCP-1/CCL2 are all critical regulators of cancer progression and metastasis via divergent mechanisms that act locally or remotely. The galectin-mediated increase of these cytokines/chemokines in various

cancers therefore is likely to make an important contribution to cancer progression and metastasis.

It is noted that although galectins-2, -4 and -8 have each been shown to induce secretion of IL-6, G-CSF, GROα/CXCL1 and MCP-1/CCL2 by vascular endothelial cells *in vitro*, direct correlation of serum galectin concentrations in cancer patients was observed predominantly with G-CSF and IL-6 and there was less or no correlation with MCP-1/CCL2 and GROα/CXCL1. It is possible that the galectin-mediated secretion of GROα/CXCL1 and MCP-1/CCL2 may be a secondary effect consequent on galectin stimulation of IL-6 and G-CSF secretion. Both GROα/CXCL1 and MCP-1/CCL2 have been shown previously to be inducible by pro-inflammatory cytokines. For example, IL-1 or TNFα can induce the secretion of GROα/CXCL1 in cancer as well as cancer stromal cells (435, 436) and IL-6 can induce the secretion of MCP-1/CCL2 in tumor cells (437). The observation that these galectins had no significant effect on GROα/CXCL1 levels when injected directly into the animal tail vein is in keeping with this possibility. Further investigation to determine the identity and nature of the galectin-binding ligands on endothelial cells that are responsible for the galectin-mediated secretion of these cytokines should help us to understand the actions of these galectins.

Thus, the increased circulation of galectin-2, -4 and -8 seen in cancer enhances endothelial secretion of G-CSF, IL-6, GROα/CXCL1 and MCP-1/CCL2 in the blood circulation. As these cytokines/chemokines are important promoters in cancer progression and metastasis via divergent mechanisms locally and remotely, the

galectin-mediated increase of these cytokines/chemokines in cancer patients probably has a profound influence on cancer progression and metastasis and represents a good target for cancer therapy.

# CHAPTER 7 Summary of the main findings

- Incubation of recombinant galectin-3, at concentrations found in sera from cancer patients, with HMVEC-Ls enhances subsequent adhesion of MUC1-negative cancer cells to HMVEC-Ls.
- The supernatant from galectin-3-treated HMVEC-Ls, when incubated with cancer cells, promotes their adhesion to endothelial cells.
- Galectin-3 shows binding to several ligands expressed by HMVECs.
- Galectin-3 at pathological concentrations induces dose- and time- dependent secretion of four cytokines (G-CSF, GM-CSF, IL-6 and sICAM-1) from blood vascular endothelial cells *in vitro*.
- The galectin-3-induced secretion of cytokines enhances the endothelial expression of cell surface adhesion molecules including integrin $\alpha_5\beta_1$, E-Selectin, ICAM-1 and VCAM on the cell surface of HMVEC-Ls.
- The galectin-3-induced secretion of cytokines promotes endothelial cell migration and tubule formation in angiogenesis.
- Intravenous introduction of a pathological circulating galectin-3 concentration into nude mice resulted in significant increase of G-CSF, GM-CSF, IL-6 and sICAM-1 concentrations in the blood circulation within 48 hr.
- Direct injection into nude athymic mice of a combination of these four cytokines at galectin-3-induced levels increased lung metastasis of MUC1-negative human melanoma ACA19$^-$ cells.

- Galectin-3 concentrations are significant correlated with G-CSF, IL-6 and sICAM-1 levels, but not with GM-CSF level, in colon cancer patients with metastasis.
- Galectin-3 also shows binding to cancer cells and induces tyrosine phosphorylation.
- The presence of galectin-2, -4 or -8 as well as the supernatant from galectin-2, -4 or -8 treated HMVEC-Ls induces cancer cell adhesion to endothelial cells.
- The presence of galectin-2, -4 or -8 also stimulates secretion of G-CSF, IL-6, GROα and MCP-1 from HMVEC-Ls.
- Galectin-4 and -8 induce secretion of G-CSF, IL-6, GROα and MCP-1 in dose- and time-dependent manners, and galectin-2 induces secretion of G-CSF, IL-6 and GROα in dose- and time-dependent manners.
- The secretion of these cytokines in response to these galectins enhances the expression of endothelial expression of cell surface adhesion molecules integrin $\alpha_5\beta_1$, CD44 or VCAM, causing cancer cell adhesion to vascular endothelium.
- The galectin-induced secretion of these cytokines also enhances endothelial tubule formation in angiogenesis.
- The secretion of cytokines induced by galectins can be inhibited by the presence of lactose.
- Intravenous injection of a pathological circulating concentration of galectin-2, -4, -8 separately or a combination of galectin-2, -4 and -8 together into nude mice resulted in increase of G-CSF, GM-CSF, IL-6 and sICAM-1 concentrations in the blood circulation within 24 or 48hr.

- Regression analysis showed significant correlation of G-CSF and IL-6 levels

with each of galectins-2, -4 and -8 in the sera of breast and colon cancer patients.

# CHAPTER 8 General discussion and implications for future studies

## 8.1 Discussion

The results presented in this thesis show that galectin-3, as well as galectins-2, -4 and -8 induce secretion of various cytokines from blood vascular endothelial cells *in vitro*. The secretion of these cytokines enhances the endothelial expression of cell surface adhesion molecules, promoting increased adhesion of cancer cells to the blood vascular endothelium. The galectin-induced secretion of these cytokines also promotes endothelial cell migration and tubule formation in angiogenesis. These *in vitro* findings were strongly supported by the demonstration of increased concentrations of these cytokines in the sera of nude mice in response to intravenous injection of recombinant galectins and were further supported by demonstration of correlation between the levels of these circulating galectins and cytokines in the sera of human colon cancer and breast patients. A general model of the action of the galectin-endothelial interaction and subsequent cytokine secretion in metastasis promotion is shown in Fig 8.1.

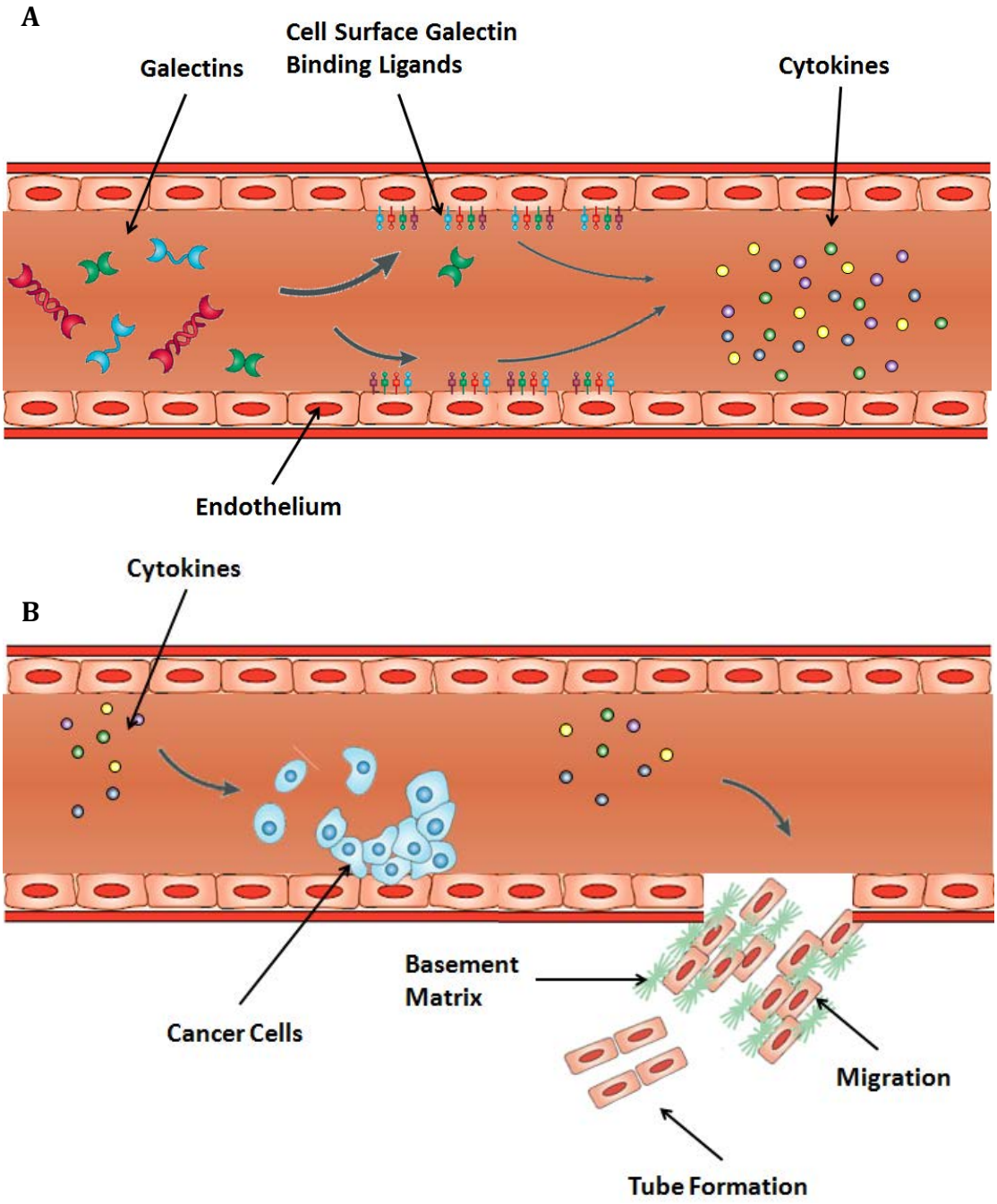



**Fig 8.1: The postulated role of interaction between circulating galectins and vascular endothelium and subsequent secretion of cytokines in metastasis promotion**

A: Galectin-endothelial interaction induces endothelial secretion of cytokines. B: the galectin-induced secretion of cytokines promotes angiogenesis and metastasis. Galectin-induced cytokines released from the endothelium induce increased expression of cell surface adhesion molecules by the endothelium and promote metastasis by increasing heterotypic adhesion and angiogenesis.

It is known that cytoplasmic galectin-3 functions as an apoptosis inhibitor by suppressing mitochondrial depolarization and preventing the release of cytochrome C (221), while nuclear galectin-3 acts as an mRNA splicing promoter (280), and in contrast to cytoplasmic galectin-3, has pro-apoptotic activity (240). Cell surface-associated galectin-3 is involved in many cell-cell and cell-matrix interactions (306, 438) and promotes cancer development and metastasis (439). Cancer cell-associated galectin-3 has been shown to contribute to tumor cell heterotypic adhesion to endothelium (315) and cancer cell invasion (262, 263) and the cytokine-mediated effects demonstrated in this thesis add to these findings.

Galectin-3, the only chimeric galectin, is also unique in the galectin family for containing tandem repeats of short amino-acid stretches fused onto the CRD (440, 441). However, all members of the galectin family are defined by their ability to recognize β-galactose and by their consensus amino-acid sequences (170). The carbohydrate binding sites of galectins can accommodate adjacent saccharides as well as galactose and different galectins are specific for different oligosaccharides (177, 178). The studies reported here show that galectin-2, -3, -4 and -8 at pathological concentrations seen in cancer patients induce secretion of several metastasis-promoting cytokines by interaction with the vascular endothelium. These cytokines then increase endothelial expression of several cell surface adhesion molecules that enhance cancer cell adhesion to the endothelium in metastasis.

The patterns of cytokine secretion induced by each galectin member have been shown not to be the same. For example, galectin-3 induces a significant increase of

$\alpha_5\beta_1$, but not $\alpha_5\beta_3$ integrin. Integrin $\alpha_5\beta_3$ is known to be recognized by a wide range of ECM molecules, including fibronectin, fibrinogen, von Willebrand factor, vitronectin, and proteolysed forms of collagen and laminin, whereas integrin $\alpha_5\beta_1$ is shown to be selectively bound by fibronectin (442). The different patterns of the changes of the cell surface adhesion molecules in response to different galectins may aid future identification of the exact molecular mechanism of the galectin-induced secretion of cytokines.

Serum levels of cytokines are higher in various types of cancers than in healthy people and people with benign tumors (443-452). Cytokines released in response to infection, inflammation and immunity, can inhibit tumor development and progression. On other hand, host-derived cytokines can promote cancer growth, attenuate apoptosis and facilitate tumor cell invasion and metastasis (96). Cytokines can have either pro- or anti- inflammatory activity and immunosuppressive activity, depending on the microenvironment. Immune cells are the major source of cytokines but many human cells are capable of producing them during cancerous conditions (453, 454). Efforts to understand cytokine function during tumor development and progression are complicated by the pleiotropy of the cytokine action and also by the ways in which the microenvironment influences the effects of individual cytokines (455). In some instances cytokine release has correlated with tumor suppressing effect. For example the historical administration of “Coley’s toxins”, bacterial extracts administered as cancer immunotherapy, was shown to result in marked increase in cytokine levels and tumor clearance in some of the treated patients (456, 457).

Subsequent studies about systemic infusion of individual cytokines were conducted to retain the therapeutic potential of Coley's toxins as well as ameliorate the undesirable side effects (458-460) but with, at best, only modest therapeutic benefits.

## 8.2 Future studies

The endothelial cell surface ligands for each galectin that are responsible for subsequent stimulation of cytokine secretion remain unknown. Although IL-6 is shown to be induced by each of these galectins, a different range of cytokines is induced by each galectin. This indicates that although these galectin members may share similar endothelial cell surface ligands in their induction of cytokines, each of them likely also has its own specific ligands.

Furthermore, although the galectin-induced cytokine secretion is inhibitable by lactose and TF-expressing glycans, it remains to be tested whether the galectin-endothelial interaction in galectin-induced cytokine secretion is mediated through protein-carbohydrate or protein-protein interactions. And if galectin-induced cytokine secretion is indeed mediated by protein-carbohydrate interaction, what is the nature of the glycan ligands. Future investigations in these areas will help greatly to clarify the molecular mechanisms of galectin-mediated cytokine secretion.

The studies reported here provide further evidence for the significance of the increased circulation of galectins in metastasis promotion. They also indicate that

targeting the action of these circulating galectins may represent a promising novel therapeutic strategy to reduce metastasis and improve cancer survival. The design and development of galectin inhibitors may have great therapeutic potential.

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# Published Work

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Clinical Cancer Research 2013 Mar; doi: 10.1158/1078-0432. CCR-12-2940

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British Journal of Cancer 2014 Feb; 110(3):741-52; doi: 10.1038/bjc.2013.793.